A study of the effects of exposure to a carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine, in bacteria and mammalian cells

by

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TO MARGARET, MY WIFE.

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Miss Margaret Nicol typed this manuscript; I am most appreciative of the time and care which she has given to the execution of this task.

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Abbreviations

The biochemical abbreviations used in this thesis are those recommended by the Editorial Board of the Biochemical Journal, in their Instructions to Authors (Biochem. J. (1967) 102, 1). The abbreviations used to describe bacterial genotype, or phenotype, are those recommended by Demerec, Adelberg, Clark and Hartman (1966). The following are also used:-

ALA	δ aminolaevulinic acid.
AFV	bovine albumin, fraction V.
BSS	balanced salt solution.
BCNU	1, 3-Bis-(2-chlorethyl)-1-nitrosourea.
DMN	dimethylnitrosamine.
EDTA	ethylenediaminetetracetic acid.
G_1, S, G_2, M and G_0	phases of the cell division cycle.
GSH	reduced glutathione.
HN2	nitrogen mustard (allyl isothiocyanate).
HS2	sulphur mustard (bis-(2-chlorethyl)-sulphide).
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine.
MNU	N-methyl-N-nitrosourea.
MNUE	N-methyl-N-nitrosourethane.
MU	methylurea.
mRNA	messenger ribonucleic acid.
rRNA	ribosomal ribonucleic acid.
tRNA	transfer ribonucleic acid.
TCA	trichloracetic acid.
TdR	(deoxy)thymidine.

All temperatures are given in degrees Centigrade.

PART I

GENERAL INTRODUCTION

I. GENERAL INTRODUCTION

The study of living processes, unicellular and multicellular, has been remarkably unified by the general understanding of cellular metabolism and genetics at a molecular level. This has been made possible by the comprehension that nucleic acids are of fundamental importance in such processes. An outline of the work which has led to this understanding and to present day concepts of their function must be given.

Material isolated from the nuclei of pus cells by Miescher (1869) and similar substances extracted from animal tissue and yeasts by Altmann (1889) were shown, by analysis of their degradation products, to be composed of sugar, phosphoric acid and a mixture of the purine bases, adenine and guanine, with the pyrimidine bases, cytosine, thymine or uracil. The observation that the sugar was deoxyribose in animal cells and ribose in plant cells prompted the belief that herein lay the difference between plants and animals. However, subsequent refinements of extraction and cytochemistry revealed that the deoxyribonucleic acid (DNA) was confined to the nucleus, in particular the chromosomes, whilst the ribonucleic acid (RNA) was cytoplasmic.

General opinion did not favour a substance composed of four nitrogenous bases, of sugar and of phosphate to comprise the informational content, or "genes", of a cell. Instead, the more complex and varied molecule of protein was preferred. Even the studies of Avery, MacLeod and McCarty did not receive a great deal of attention. These authors reported (1944) that a substance extracted from heat killed pneumococci (type III), which had the characteristics of DNA and was free of protein, transformed "rough", unencapsulated variants of pneumococci (type II) into capsulated, smooth colony forming cells of type III.

A. Deoxyribonucleic acid: structure and function.

A considerable stimulus to thoughts on the nature of the genetic determinant was provided by the model proposed by Watson and Crick (1953) for the physical structure of DNA, which was based on available analytical data of stereochemical considerations and X-ray diffraction studies. It comprised a double, right-handed helix consisting of a back-bone of alternate sugar and phosphate molecules, the chains being held in close association by hydrogen bonds between the nitrogenous bases of the nucleotide units (see Fig. 1). The proposed pairing of the bases, adenine (A) with thymine (T) and guanine (G) with cytosine (C), on the grounds of hydrogen bonding ability, would give a regular structure to the helix. The observation of Chargaff (1955) on the equivalence of the molar proportions of the purines and pyrimidines (A+G = C+T), and of amino bases with the keto bases (A+C = G+T) in DNA was in agreement with this model.

A means of replication of this DNA in the genetic determinant was envisaged if the strands were separated and the nucleotides re-associated with new nucleotide units, along pairing principles outlined above, to give rise to two identical "daughter" molecules, each composed of one old and one new polynucleotide strand (see Fig. 2). This "semi-conservative" mode of replication was given experimental support by Meselson and Stahl (1958). These authors examined the density gradient centrifugation patterns of DNA extracted from Escherichia coli, which was grown initially in medium that contained "heavy" ^{15}N and then harvested at intervals after transfer of cultures to medium that contained only ordinary nitrogen isotope, ^{14}N. The data, examined in the knowledge of population doubling times, indicated that the DNA of daughter cells was composed of one strand from the parent and one newly synthesised strand. Enzymes which catalysed the synthesis of DNA in vitro were subsequently described (Kornberg, Lehman, Bessman and Sims, 1956,) and are discussed in greater detail later (III, A.4.e).

The molecule of DNA was therefore demonstrated to satisfy the criteria required of a determinant of heredity, in stability of structure and the ability to be replicated. The capacity to contain information was disclosed by studies with RNA, in relation to the synthesis of proteins, and by the development of transformation studies in bacteria,

Figure 1. Structure of the DNA molecule.

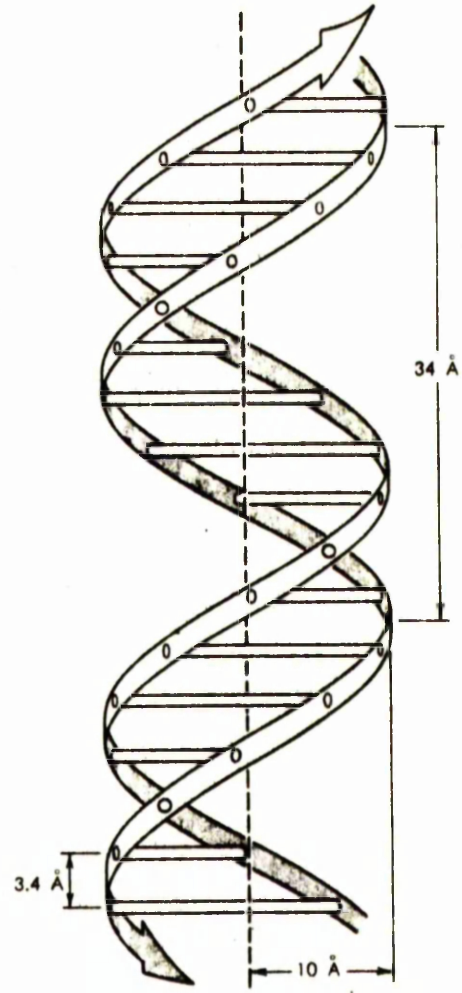
(a) Diagrammatic representation of a DNA molecule as a double helix.

(from Braun, 1965: after Watson and Crick, 1953.)

(b) Diagrammatic representation of DNA chemical structure, to show alternating units of deoxyribose and phosphate (the "backbone") and hydrogen bonding between base pairs. A, C, G and T are defined in the text.

(from Kornberg, Scientific American, October, 1968.)

(a)



(b)

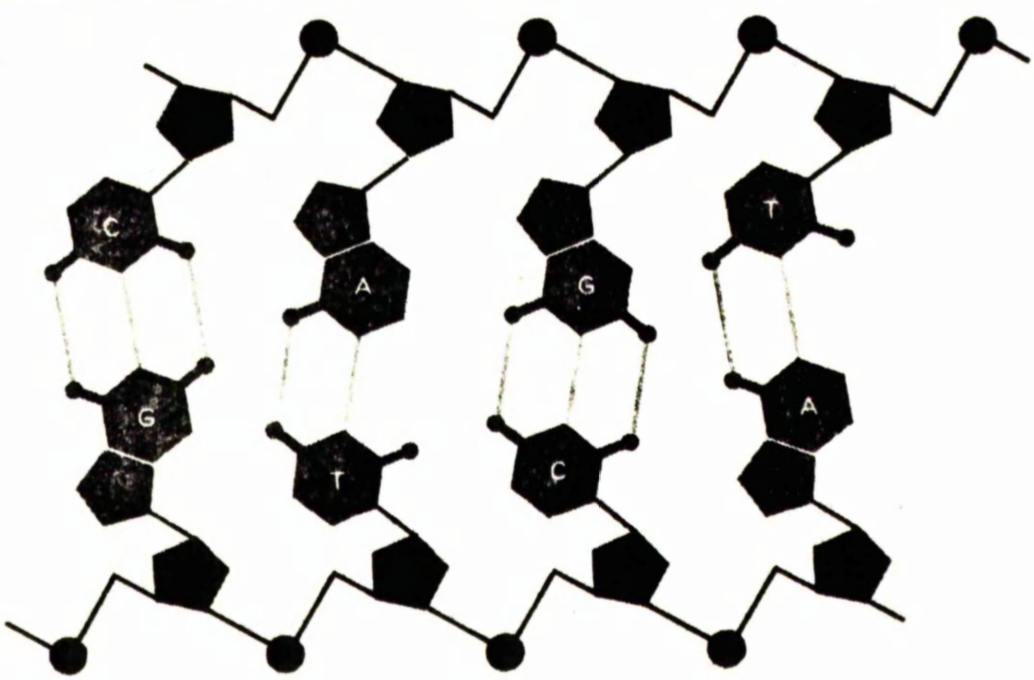
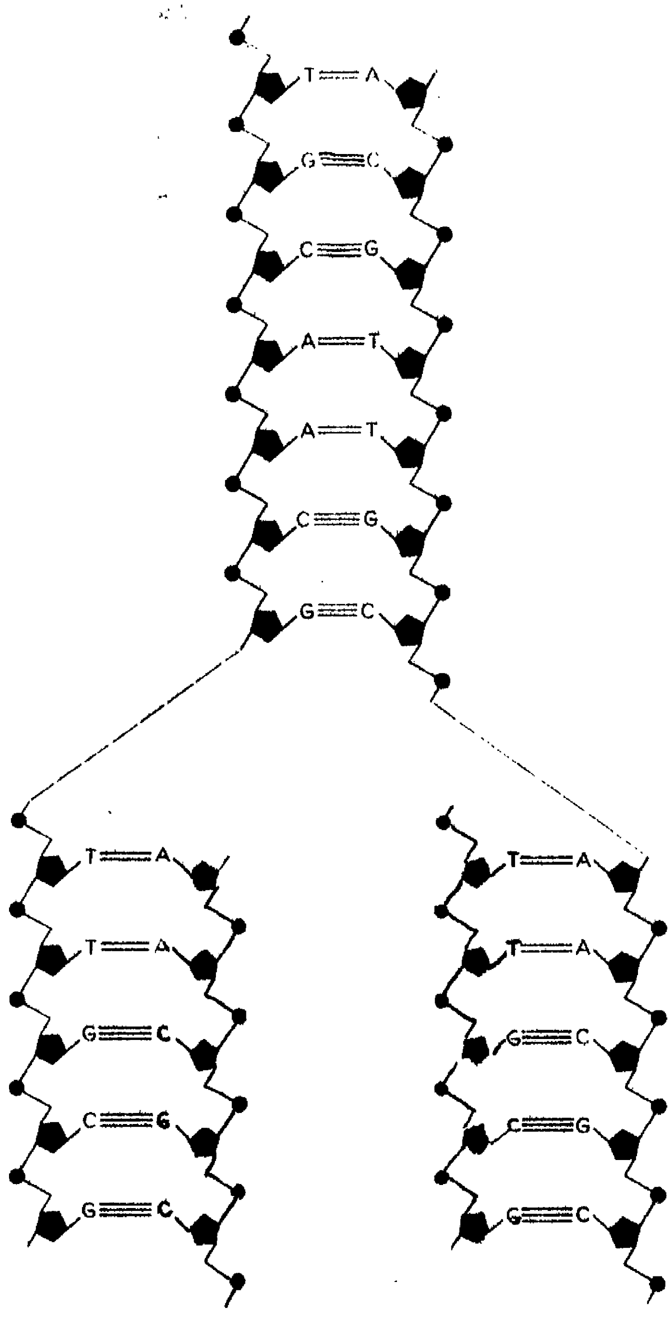


Figure 2. Replication of the DNA molecule.

Diagrammatic representation of a DNA molecule to show separation of strands to give two "new" daughter strands, by association of fresh nucleotide units, along pairing principles outlined in the text, with the individual strands of the parent molecule.

(from Kornberg, Scientific American, October, 1968.)



T A
G C
C G
A T
A T
C G
G C
T A
T A
G C
C G
G C
T A
T A
G C
C G
G C

based on the observations of Avery, MacLeod and McCarty (1944) already noted. Transformation has since been demonstrated in a number of bacterial species, in which the transfer of such characters as drug and antibiotic resistance, antigenic structure and the synthesis of specific enzymes has been studied (see Braun, 1965).

The DNA molecule within the bacterial cell was shown by Cairns (1962, 1963) to have a circular structure on autoradiography of material very gently extracted from Escherichia coli after lysis of cells. The molecular weight of this DNA was estimated to be 2×10^9 daltons and was considerably in excess of the value of 10^6 daltons reported for DNA extracted by earlier techniques (Marmur, 1961). A circular structure was also proposed, for example, for the DNA of bacteriophage øX 174 (Fiers and Sinsheimer, 1962). Hotta and Bassel (1965) isolated large molecular weight fragments of DNA from mammalian cells by gentle procedures of extraction, although the population of DNA fragments was very heterogeneous with regard to size. In mammalian chromosomes the DNA is intricately associated with protein (Zubay, 1964) which complicates extraction in the pure, undegraded state. However, a circular appearance, on electron micrographs, of mitochondrial DNA from mouse fibroblasts has been reported (Nass, 1966).

B. Ribonucleic acid, proteins and the transfer of genetic information within the cell.

The evidence for association between RNA and protein synthesis originates from the observation (Caspersson, 1950; Brachet, 1950) that RNA was particularly abundant in cells engaged in the synthesis of protein. Enucleated (essentially DNA-free) algae and amoebae continued to synthesise protein for a prolonged period of time, which thus indicated that DNA was not the direct template for protein, and that there must be an intermediary molecule. Evidence that RNA was that intermediate accumulated convincingly.

1. Separation of ribonucleic acid fractions.

The RNA extracted in bulk from cells (e.g., Escherichia coli) separated into three distinct fractions when it was subjected to analysis by, for example, sucrose gradient centrifugation or chromatography on methylated albumin coated on kieselguhr.

The smaller, low molecular weight fraction (sedimentation value, 4S, molecular weight around 25,000 daltons and equivalent to a molecule of approximately 70-80 nucleotides) was correlated with the RNA found in the supernatant after centrifugation of disrupted cells. It was shown (Hoagland, Zamecnik and Stephenson, 1957) to be involved in the transfer of amino acids to the sites of protein synthesis and given the name transfer RNA (tRNA). The structure and function of this type of RNA will be discussed again later. (I, B.3.c.).

The heavier, larger molecular weight fraction had two components, of which the sedimentation values were 16S and 23S. These components corresponded to the RNA of the basic units, designated 30S and 50S respectively, into which the ribonucleoprotein particles of the cytoplasm could be separated (Tissières, Watson, Schlessinger and Hollingworth, 1959). These ribonucleoprotein particles, known as ribosomes, were found to contain about 50% protein and 50% RNA (Arnstein, 1963); the RNA component was known as ribosomal RNA (rRNA). The ribosomes will be discussed later (I, B.3.a.) in relation to the cellular components involved in protein synthesis.

From the ribosomes it was possible to isolate, at low Mg^{2+} concentrations, a further unit of RNA with sedimentation values of 8-12S. It was found initially in *Escherichia coli* infected with bacteriophage (Nomura, Hall and Spiegelman, 1960), but was later isolated from uninfected *E. coli* (Hayashi and Spiegelman, 1961) after the cells had been subjected to treatment of "nutritional stepdown" by transfer from rich to basic medium. The continuation of growth after such treatment implied the function of new biosynthetic pathways, for which enzymes would be required. This unit of RNA was called "informational" RNA but is now known as messenger RNA (mRNA). Its function is discussed later (I, B.3.a.).

The proportions of these various types, or species, of RNA in material extracted from bacterial cells was of the order of 80% rRNA, 15% tRNA and 5% mRNA. Mammalian cell RNA showed a similar separation. The sedimentation values of the heavier components showed greater spread, however, which reflected the material of origin and the method of isolation (Davidson, 1965).

2. Synthesis of ribonucleic acid transcription.

Ribonucleic acid, as mentioned earlier, was shown to be composed of purine and pyrimidine bases, ribose and phosphate. It differed from DNA, however, not only in the sugar molecule but also in its constituent bases, which were adenine (A), guanine (G), cytosine (C) and uracil (U); there was no thymine (T). Enzymatic synthesis of RNA has been achieved with cell free preparations from bacteria (Ochoa, Burma, Kröger and Weill, 1961) as well as from animal cells (Burdon and Smellie, 1962). The process was catalysed by the enzyme, DNA-dependent RNA polymerase (E.C.2.7.7.6., Nucleosidetriphosphate:RNA nucleotidyltransferase) which utilised DNA as the primer (template) for the synthesis of new polyribonucleotide material, with the triphosphates of adenosine, guanosine, cytidine and uridine as substrates. The polyribonucleotide product of this enzyme had a composition, in terms of constituent nucleotides, which reflected that of the DNA primer used. The results indicated a "complementary" pairing phenomenon, thus: deoxyadenine with uridine, deoxyguanine with cytosine, deoxycytosine with guanine and deoxythymidine with adenine. Many examples of this complementarity of base composition between DNA primer and the RNA products of RNA polymerase action have been given by Smellie (1965).

Further evidence of complementarity of structure between the DNA primer and its product has been given by the molecular "hybridisation" techniques of Geidushek, Nakamoto and Weiss (1961). These experiments tested the ability of the reaction product (RNA) to interact, under suitable conditions, with "denatured" single strand DNA molecules to form ribonuclease-resistant DNA-RNA hybrids, signifying complementary base sequence between the greater part of the two strands. Similar hybridisation techniques were used by Hayashi and Spiegelman (1961) and by

Ritossa and Spiegelman (1965) to demonstrate for mRNA and rRNA, respectively, a base sequence complementary to the DNA of the parent cell. The process of synthesis of RNA as a sequence of bases complementary to DNA was referred to as transcription. The significance of transcribed mRNA and rRNA in the transmission of genetic information within the cell was understood from studies of the cell structures involved in protein synthesis.

3. Ribonucleic acid and the synthesis of protein: translation.

The process by which the sequence of ribonucleotides of RNA, transcribed from DNA, determined the sequence of amino acids in a polypeptide chain was called translation. It became understood from consideration of the cellular components, and their molecular structure, involved in the synthesis of proteins, which are composed of polypeptide chains.

a. Cellular components involved in polypeptide synthesis.

The association of ribosomes into aggregates, called polysomes, was deduced from ultracentrifugational studies of the ribosome particles obtained by gentle disruption of cells, for example, reticulocytes (Warner, Knopf and Rich, 1963). After brief incubation of rabbit reticulocytes with ^{14}C -labelled amino acids, the labelled polypeptide was found to be associated with the polysome fraction of the cells, whereas there was little or no radioactivity with the monoribosome fraction (Warner et al., 1963). As a result of this and subsequent work, Rich (1963) and his colleagues proposed that the ribosomes were linked together by strands of mRNA. This species of RNA (mRNA) had already been demonstrated to possess a base sequence complementary to the DNA of the cell in which it was produced (Hayashi and Spiegelman, 1961).

The major protein structure manufactured by reticulocytes was assumed to be the polypeptide chain, of approximately 150 amino acids, to be incorporated into the molecule of haemoglobin. On the hypothesis (I, B.3.b.) that three nucleotides were required for each amino acid, the length of polyribonucleotide (mRNA) required to direct the synthesis of such a polypeptide was calculated. Measurements, on electron microscope pictures, of polysome strand length of preparations from reticulocytes agreed with the theoretical predictions (Slayter, Warner, Rich and Hall, 1963). The purpose of the attachment of ribosomes (rRNA and protein) to mRNA appeared, therefore, to be related to the translation of the message contained in the nucleotide sequence of mRNA.

b. Translation and the genetic code.

If the sequence of nucleotides of mRNA determined the sequence of amino acids in a polypeptide, it was argued that only permutations of three out of the possible four nucleic acid bases (A, C, G and T), to give a total of 64 alternatives, provided a sufficient number of individual "codes" for the twenty amino acids. Work by Crick (1962) and his colleagues with mutants of bacteriophage T4, and by Nirenberg and Matthaei (1961) and Ochoa (1964), with in vitro protein synthesising systems that utilised synthetic polyribonucleotides, provided convincing experimental evidence for a triplet code. That is to say, a sequence of three nucleotides of mRNA, transcribed from DNA, specified for the insertion of one particular amino acid into a polypeptide chain. Specific features of the code, such as the existence of more than one triplet code for a given amino acid, or triplet codes for polypeptide chain termination and other aspects of "punctuation" of the genetic message, have been reviewed by Woese (1967). Analysis of amino acid sequence in the protein of cytochrome c from a variety of sources revealed regions of identity (Jukes, 1966), which supported the possibility that the code was universal for all cells.

c. Intracellular transport of amino acids.

The combination of mRNA and ribosomes into a unit, as outlined above, which was active in the formation of polypeptide, was incomplete without a mechanism for the transport and selection of amino acids for incorporation into polypeptide. This deficiency was remedied by the study of the small molecular weight molecules of tRNA (Hoagland, Zamecnik and Stephenson, 1957; Zamecnik, 1962). Association of this RNA species with the unit of polypeptide synthesis, the polysome, was supported by the report that a binding site for tRNA molecules was present on the 50S subunit of the ribosomes (Cannon, Krug and Gilbert, 1963). Investigations of the heterogeneity of tRNA preparations, as, for example, on separation by electrophoresis (Richards and Gratzer, 1964) led to the belief that each amino acid might have its own specific tRNA. The evidence for this was reviewed by Zamecnik (1962), and indeed it was demonstrated that more than one tRNA might exist for a given amino acid, for example, leucine (Apgar and Holley, 1964).

The essential features of this small polyribonucleotide (tRNA) were noted to be a high content of unusual bases, for example, pseudouridine, and a high degree of secondary structure. From their study of the base sequence, a clover-leaf configuration was proposed by Holley et al. (1965). The terminal sequence of nucleotides, pCpCpA, which was common to all tRNA molecules, was important for the binding of amino acid to be transported to the ribosomes. A nucleotide sequence site, or anticodon, which "recognised" the appropriate amino acid triplet codon of mRNA was also proposed (Holley et al., 1965).

Further information on the structure of this small molecule is expected to follow the report of the crystallisation of tRNA by Clark et al. (1968).

C. Regulation of genetic expression.

The mechanism by which genetic expression is regulated in cells is not known. The association of the basic protein, histone, with DNA, as nucleohistone, in the chromatin of the cell nucleus has invited speculation as to its function. Zubay (1964) discussed the relation of histones to the regulation of RNA and protein synthesis, in addition to their importance to the structure of DNA in the chromosome. The possibility was considered that the histone protein helix lay in the broad groove of the DNA double helix. Control of protein synthesis was perhaps exercised by the prevention of the transcription (in the broad groove), to RNA, of segments of the DNA chain. Experimental support for this proposal was provided by Bonner and Huang (1963) who observed a decreased ability of chromosomal nucleohistone to promote *in vitro* synthesis of RNA in comparison to DNA essentially free of protein.

A theory of gene control was proposed by Jacob and Monod (1961) to explain the results of a genetical analysis of bacterial mutants. The various strains carried mutations, i.e., stable and heritable alterations, in the control of the synthesis of the "lactose" enzymes, β-galactosidase, β-galactosidase-permease and β-galactosidase-acetylase. The theory, shown diagramatically, Fig.3, implied that a repressor molecule, most probably protein, coded by a separate region of DNA, interacted with a site on the gene, defined as the "operator". Attachment at this site prevented the transcription of the DNA segment which coded for the structure of the enzymes. The composite unit of the operator and structural genes was referred to as the operon.

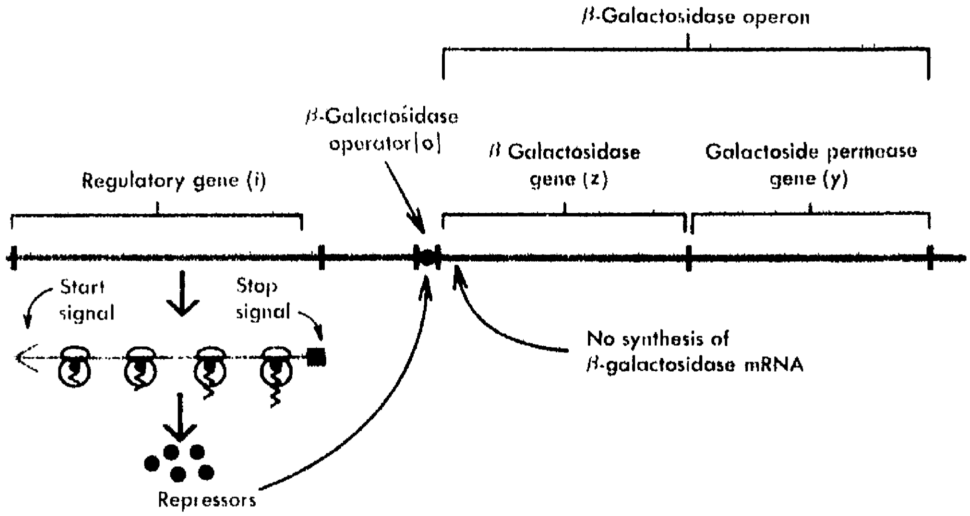
Monod, Changeux and Jacob (1963) suggested that, for a protein (enzyme) with two or more active sites, reaction at one could interfere with the affinity of the other for its substrate. Inhibition by this means was defined as "allosteric". It was possible that inducing agents (e.g. enzyme substrate) acted as allosteric inhibitors of the repressor molecule such as to alter its reaction with the operator region to block transcription. In the same fashion, inhibitors (e.g. reaction end-product) reacted with repressor molecule to increase affinity for the operator region. Indeed, a protein repressor molecule has been isolated from *Escherichia coli* (Gilbert and Müller-Hill, 1967).

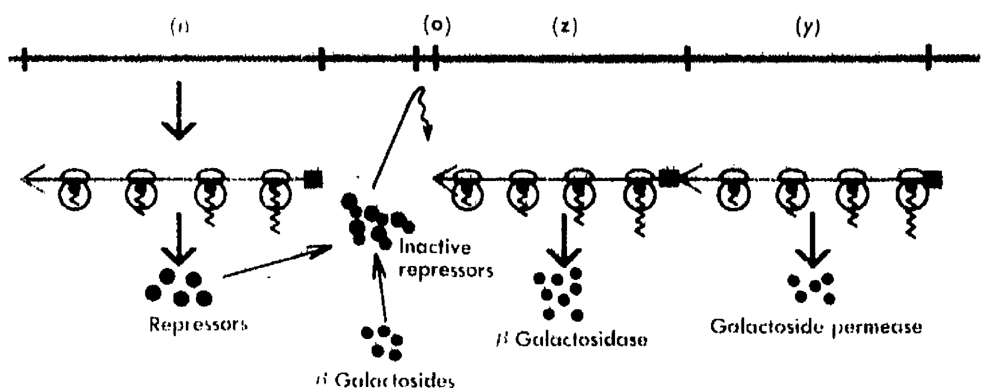
This model did not account for all the observed phenomena of biosynthetic control. Alternative proposals have been made, for example, premature detachment of ribosomes from mRNA (Ames and Hartman, 1963) or inactivation of particular tRNA molecules (Stent, 1964). Technical limitations have prevented direct tests of these alternatives (Brenner, 1965). Evidence in support of the Jacob-Monod theory has been reviewed by Bretscher (1968).

It is probable that the regulation of genetic expression in mammalian cells is more complex.

Figure 3. Theory of gene regulation according to Jacob and Monod (1961).

(for the β-galactosidase operon of E. coli, from Watson, 1965.)


β-Galactosidase operon
β-Galactosidase operator (o)
β Galactosidase gene (z)
Galactoside permease gene (y)
Regulatory gene (i)
Start signal
Stop signal
No synthesis of β-galactosidase mRNA
Repressors

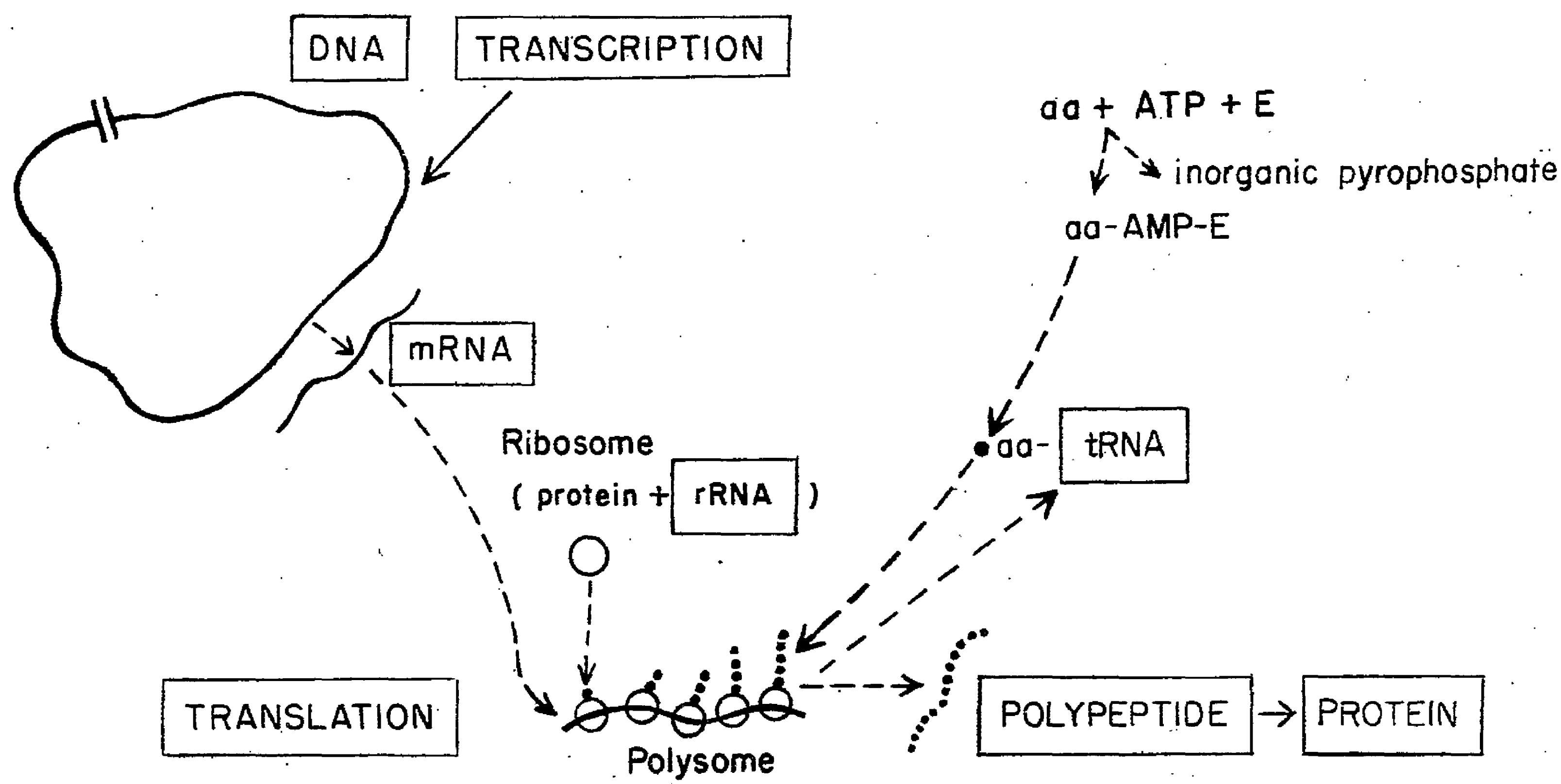

(i)
(o)
(z)
(y)
Inactive repressors
Repressors
β Galactosidase
Galactoside permease
β Galactosides

D. Function of the nucleic acids: Summary.

An outline of the work which has contributed to the appreciation of the significance of nucleic acids in relation to biological studies has been given above. The general current opinion is that hereditary information is stored in the DNA of the cell as a sequence of nucleotide units, which is transcribed into RNA, as mRNA. This mRNA is translated on the ribosomes (particles of rRNA and protein) to direct the incorporation, into polypeptide chains, of individual amino acids bound to tRNA molecules for transport to the ribosomes. This inter-relationship between DNA and the different species of RNA, the evidence for which has been detailed in the above paragraphs, is presented diagrammatically in Fig. 4. The site and mechanism of the secondary and tertiary structural alterations to polypeptides in the formation of the protein molecule has not been determined. It must also be noted that the amino acid requires enzymic activation, involving ATP, prior to its attachment to the molecule of tRNA for subsequent transport and incorporation into a polypeptide chain.

Figure 4. Diagrammatic representation of some major steps in the function of DNA and RNA in the synthesis of protein.

(from Braun, 1965).


DNA
TRANSCRIPTION
aa + ATP + E
inorganic pyrophosphate
aa-AMP-E
mRNA
Ribosome
(protein + rRNA)
aa- tRNA
TRANSLATION
Polysome
POLYPEPTIDE
PROTEIN

PART II

STUDIES WITH BACTERIA

II. STUDIES WITH BACTERIA

A. Introduction.

The suitability of bacteria as experimental cell systems in which to examine the properties and function of the nucleic acids is apparent from the references to such work in the preceding paragraphs. In particular, the analysis of bacterial mutants (i.e., bacteria showing persistent, transmissable alterations of character not evident in the parent) has provided valuable information on the genetic factors determining, for example, biosynthetic pathways or antibiotic resistance (see Braun, 1965). Such information led Jacob and Monod (1961) and their colleagues to propose a theory of gene regulation, to which reference has already been made (I, C.). The ability to induce mutations in bacteria with different physical and chemical agents is now well recognised. General acceptance of the molecule of DNA as the hereditary determinant of cellular activity has encouraged discussion as to the changes that could be induced in this stable molecule that might account for the observed alteration of character. From the accumulated evidence (reviewed above) that the sequence of individual deoxyribonucleosides (dA, dC, dG and dT) of DNA is of primary importance to the structure and composition of proteins, and hence of enzymes, emphasis has been stressed on the way in which an alteration to this sequence might occur.

The mutagenic effect of ultraviolet irradiation (UV) for bacteria is commonly acknowledged and provides a convenient example on which to comment on possible reaction of chemical and physical agents with nucleic acid bases. Attention was given by Beukers and Berends (1961) to the ability of UV to promote the formation of a dimer of thymine (T) residues, between adjacent pairs on the same strand, or between bases of complementary strands of DNA. Release of the dimers from DNA of UV-resistant, but not from the DNA of UV-sensitive, *Escherichia coli* strains after UV irradiation (Boyce and Howard-Flanders, 1964; Setlow and Carrier, 1964) was evidence that such dimer formation might play an important rôle in the biological effects of UV *in vivo*. It was possible that the wrong nucleic acid base might be inserted by the repair process, or that excision of the offending dimer might lead to deletion of genetic material (see Fig. 5). These events might be expressed as a mutation in descendents of the treated cell.

The importance of fidelity of base pairing on replication of DNA strands is self-evident for the maintenance of genetic stability, since change or deletion of but a single nucleotide could cause significant alterations to the sequence coding for a polypeptide or even an enzyme protein. Incorporation of an incorrect amino acid into a polypeptide could alter, for example, the tertiary structure of an enzyme such as to impair, or cause the loss of, activity. A major factor determining the formation of a pair between nitrogenous bases on opposing chains of the DNA helix is the ability to form hydrogen bonds. Normally guanine pairs with cytosine and thymine with adenine but the possibility that alkylated guanine might pair anomalously with thymine was considered by Brookes and Lawley (1964) in a review of the effects of alkylating agents on DNA, in relation to carcinogenesis. On theoretical grounds the introduction of an alkyl group at N-7 would increase the ionisation of N-1 atom of the same guanine residue at neutral pH, which would favour anomalous pairing with thymine (see Fig. 6). Assuming thymine subsequently paired correctly with adenine this would lead to replacement of a GC pair with an AT pair in the DNA strands of descendents of the cell, to be expressed as a mutation. This exchange of a purine by another purine, or of a pyrimidine by another pyrimidine, is called base transition. The effect of a wide variety of different mutagenic agents on nucleic acids has been discussed in recent articles (Freese, 1963; Lawley, 1966).

The isolation of bacterial mutants from a population of organisms treated with a mutagen is simplified by the capacity of bacteria to form colonies on the surface of chemically defined solid media. The constituents of the medium can be so altered as to select for the growth of a distinctive mutant class (i.e. of the same genotype). Such selection of bacteria of a particular genotype, by colony formation on a chemically

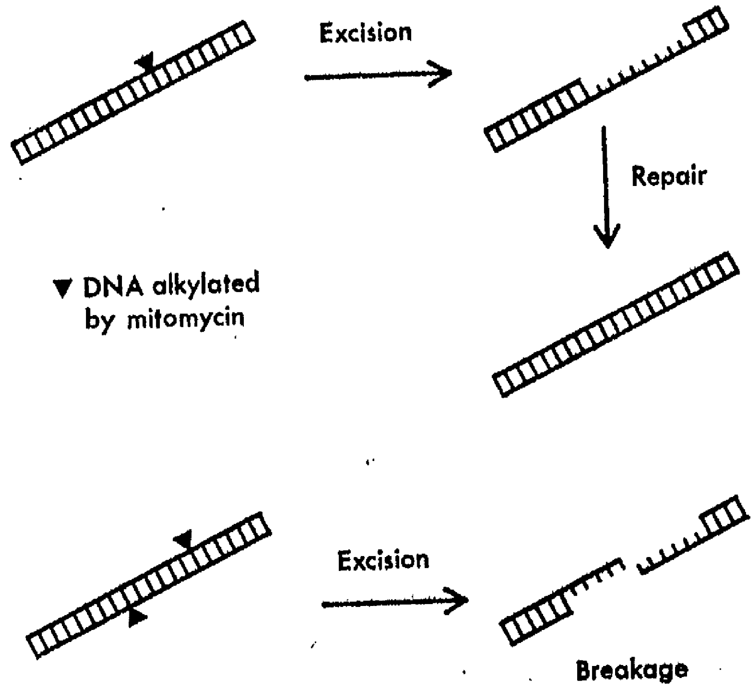


Figure 5. Diagrammatic representation to show some possible consequences of DNA alkylation (e.g. by mitomycin).

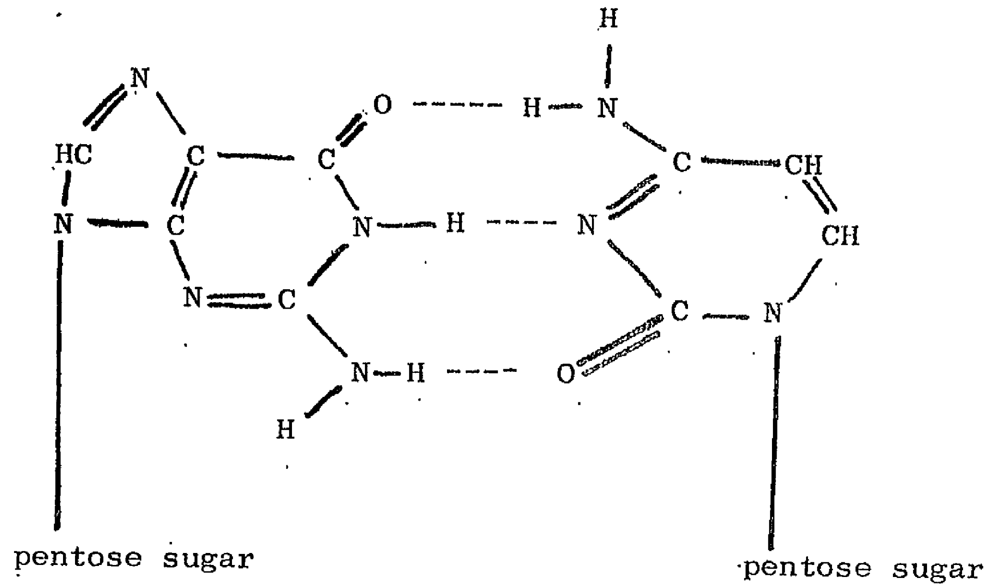
(from Collins, Brit. med. Bull., 21, 1965).

Figure 6. Hydrogen-bonding of guanine.

Normal pairing with cytosine is shown for comparison with anomalous base-pairing of ionised 7-alkylguanine with thymine.

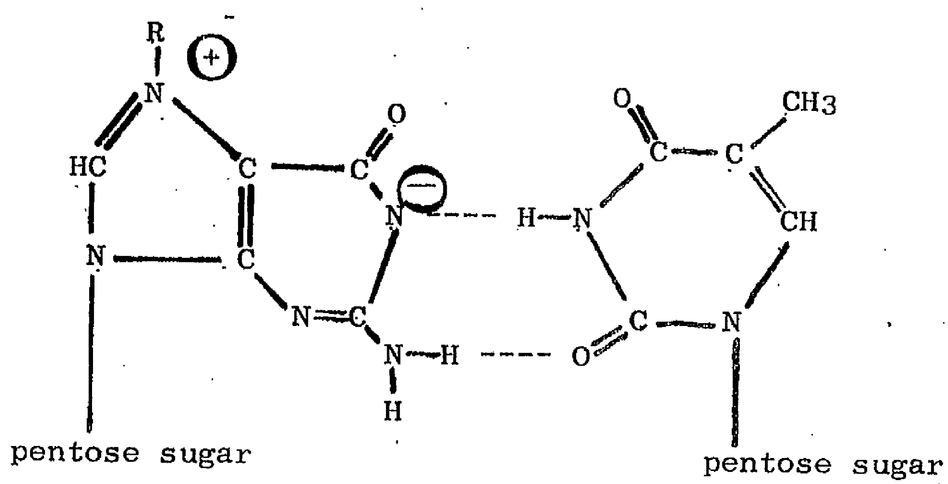
(after Brookes and Lawley, 1964.)

(a) Normal pairing



Guanine Cytosine

(b) Anomalous pairing



Ionised 7-alkylguanine Thymine

defined medium, is utilised in the experiments of bacterial transformation. The phenomenon of bacterial transformation, observed by Avery et al. (1944), may be defined as the process in which a portion of a donor cells DNA, obtained by chemical extraction, can penetrate into a related bacterial cell and replace, by a process known as recombination, a specific nucleotide sequence of the recipient's chromosome. This new nucleotide sequence is then expressed in the recipient cell through processes of transcription and translation already outlined (I, B.2 and B.3). Growth and colony formation of the "transformed" cells is selected by incubation of aliquots of diluted bacterial suspension on chemically defined medium incapable of supporting the growth of untransformed cells. This procedure allows a numerical assessment of the frequency of the transformation event.

Experiments of bacterial transformation were utilised by Spizizen (1958) to study biochemically deficient mutant strains of Bacillus subtilis. The definition of the optimal medium for the transformation reaction to occur (Anagnostopoulos and Spizizen, 1961) and refinements to the technique (Young and Spizizen, 1963) followed. A significant observation was made by Yoshikawa and Sueoka (1963) who reported that the yield of transformants, in B. subtilis strains, for a given hereditary characteristic (i.e., a "marker") was to some extent dependent on the phase of growth (stationary, early exponential or late exponential) at which the cells were harvested for extraction of DNA to be used in transformation experiments. In explanation of this difference in yield the authors suggested that there was a greater proportion of recently replicated regions of the bacterial chromosome in the different DNA extracts, the region undergoing replication being to some extent dependent on the phase of bacterial growth. To support this hypothesis the authors studied the relative frequencies of transformants, for various markers, obtained in populations of bacteria treated with different samples of DNA extracted from bacteria at different phases of growth. They showed that the values, suitably standardised, were significantly different for each marker and could be expressed as a function of the location of the marker on the chromosome, assuming a linear relationship and only one replication point per chromosome. Extensions by various authors of this principle of relationship between markers has led to a genetic map of the bacterial chromosome, on which the linear relationship of mutation sites for different biochemical pathways, and other characteristics, could be represented. Such maps are now usually represented as a circle in view of the delineation of the bacterial chromosome in this form on autoradiographs by Cairns (1963).

The linear relationship of the individual gene units to one another on the bacterial chromosome may be approximately determined in experiments of transformation from an assessment of the frequency of "linked" markers. Two markers are said to be linked when cells transformed for both markers are recovered with a frequency higher than could be expected for successive or simultaneous random transformations by distinct genetic units (see Rownd, 1965). This linkage implies proximity of the markers to each other on the bacterial chromosome. Analysis for linkage in transformation experiments may be of assistance, therefore, in the location of a particular mutant site on the chromosome.

The aim of the work reported in the first part of this thesis was to isolate haemin auxotrophs (i.e., bacterial mutants with a growth requirement for haemin) from Bacillus subtilis. Haemin is an important constituent of the compounds (e.g., the cytochromes) involved in oxidative respiration in unicellular and multicellular organisms (Lascelles, 1964). If a chemical minimal medium which supported the growth of these auxotrophs could be defined, experiments of transformation could be carried out with ..e intention of obtaining data on the location, on the B. subtilis chromosome map, of the gene locus (or loci) determining haem synthesis.

B. Materials and methods.

1. Bacterial strains

a. Parent strains of haemin auxotrophs

The origin and characteristics of the bacterial strains from which the haemin auxotrophs are derived are given in Table 1.

b. Haemin auxotrophs

The origin and characteristics of the haemin auxotrophic strains isolated in this study are given in Table 1.

c. Maintenance of stock cultures

The strains, parent and haemin auxotrophs, were maintained by weekly transfer on to fresh MD (RI 1-4) or BM (RI 5-11) and stored at 4° after initial incubation at 37° for 24 hr.

2. Bacterial cultivation

a. Media

(i) Complete: Hartley's meat digest medium (MD) was prepared according to Cruickshank (1960). Eight per cent (v/v) horse blood (Burroughs Wellcome Ltd., London) was added to MD to make blood medium (BM).

Yeast extract medium (TYE) contained in g./litre: Tryptone (Oxoid), 5; yeast extract (Oxoid), 3; di-sodium orthophosphate (anhydrous), 1,

Both MD and TYE were corrected to pH 7.6 before steam-sterilisation at 15 lbs. for 30 min.

(ii) Minimal: The medium, GGM, of Csiszár and Ivánovics (1965) was modified to contain in g./litre: sodium citrate, 3; di-potassium hydrogen orthophosphate, 2; ammonium sulphate, 1; L-glutamic acid, 0.2; magnesium sulphate (hydrated), 0.75; ferric ammonium citrate, 0.05; glycerol, 15; distilled water to 1L. Glutamic acid was converted to its monosodium salt before addition to the medium. The medium was finally adjusted to pH 7.4 before steam sterilisation at 15 lbs. for 30 min. This medium was used for the definition of minimal growth requirements of the strains and was supplemented from sterile stock solutions immediately prior

Table 1. Descriptive table of bacterial strains.

The origin and characteristics of the parent strains, and the haemin auxotrophs derived in this study, are shown.

Strain	Source	Relevant phenotype				Genotype*	Former designation
		Trp	His	Hem	Str		
RI 1	a	+	+	+	S	Prototrophic	Marburg Yale
RI 2	a	–	+	+	S	trp	168 ind$^-$
RI 3	b	–	–	+	S	trp, his	SB 25 ind$^-$his$^-$
RI 4	c (RI 2)	–	+	+	R	trp, str-1	.
RI 5	c (RI 2)	–	+	–	r	trp, str-2, hem-1	.
RI 6	c (RI 4)	–	+	–	R	trp, str-1, hem-2	.
RI 7	c (RI 4)	–	+	–	R	trp, str-1, hem-3	.
RI 8	c (RI 4)	–	+	–	R	trp, str-1, hem-4	.
RI 9	c (RI 4)	–	+	–	R	trp, str-1, hem-5	.
RI 10	c (RI 4)	–	+	–	R	trp, str-1, hem-6	.
RI 11	c (RI 4)	–	+	–	R	trp, str-1, hem-7	.

Trp = tryptophan, His = histidine, Hem = haemin, Str = streptomycin

*According to the recommendations of Demerec, Adelberg, Clark and Hartman (1966).

(a) P. Schaeffer, Institute Pasteur, Paris.

(b) S. Zamenhof, University of California, Los Angeles.

(c) Present study, with parent strain in parentheses.

(+) = synthesized, (–) = required, (S) = sensitive, (r) = resistant to 50 μg./ml., (R) = resistant to 500 μg./ml.

to use.

The minimal medium of Spizizen (1958) (see Table 2) was used, with supplements, for transformation experiments. The glucose was sterilised separately.

(iii) Solid media. Solid media, both complete and defined, contained 1.2% agar.

b. Techniques of cultivation.

Agar plates seeded with bacteria were cultured at 37^{o} unless stated otherwise. For individual colonies, bacteria were thinned out on the plates according to standard techniques with a platinum loop, or, for quantitative assessment, 0.1 ml. aliquots, delivered from suitably diluted suspensions in minimal medium, were spread with test-tubes on agar plates.

Liquid cultures were incubated at 37^{o} and aerated in a Gyrotory Shaker (New Brunswick Scient. Co., USA) at 260 cyc./min. For individual experiments, 10 ml. medium in 100 ml. Erlenmeyer flasks was used; for DNA preparation, bulk cultures in 450 ml. medium (TYE) in 2 litre flasks was used. Supplements were made to fluid minimal media as indicated by experience with minimal agar media.

c. Assessment of bacterial growth

(i) Optical density: growth of cultures in flasks with side-arms was followed by measurement of the optical density of suspensions at 640 mμ with a Bausch Lomb Spectronic 20 photometer (Bausch Lomb, Aldwych, London). Optical density values were then converted either to the number of viable colony forming units, or to dry weight of bacteria per ml. by reference to calibrated graphs.

(ii) Colony counts: the number of viable colony forming units per ml. was assessed according to techniques outlined above (II, B.2.b.). Generation time: the mean generation time of cultures was calculated according to Meynell and Meynell (1965) from colony counts of samples taken at intervals from a logarithmically growing population of bacteria, plated according to techniques outlined

Table 2. Composition of minimal medium according to Spizizen (1958).

The supplements for growth medium (GM) and transformation medium (TM), as recommended by Anagnostopoulos and Spizizen (1961), are included.

Constituent	g./litre	
$(NH_4)_2SO_4$	2	
K_2HPO_4	14	
KH_2PO_4	6	
Na citrate.$2H_2O$	1	
$MgSO_4 \cdot 7H_2O$	0.2	
Glucose	5	
Additions to give Transformation media	GM	TM
L-tryptophan	0.05	0.005
Casamino acids	0.2	0.1
$MgSO_4$ $7H_2O$	-	1.23

above (II, B.2.b.).

3. Procedure with glassware

a. Washing of glassware

(i) Flasks: Flasks and contents were first sterilised by steam at 15 lbs. for 30 min and then washed in Pyroneg solution (0.5% w/v, Diversey Ltd., Herts., England) to be rinsed thoroughly with running tap water (cold, at least three rinses). Test-tubes and metal caps were treated similarly after separation.

(ii) Pipettes: all pipettes were steeped overnight in 10% (v/v) conc. sulphuric acid and then repeatedly rinsed in an automatic syphonic rinser with running tap water before they were dried.

b. Sterilisation of glassware

Paper-capped flasks, pipettes in capped containers, test-tubes (capped) in racks, and test-tubes (spreaders) in tins were sterilised in a dry oven at 160^{o} for three hours.

4. Storage of chemicals and preparation of solutions

a. Amino acids, vitamins and nucleic acid precursors

Amino acids, vitamins and nucleic acid precursors (British Drug Houses Ltd., Poole, England) were stored at room temperature. Aqueous mixtures of vitamins (folic acid, pyridoxine, paraminobenzoic acid, nicotinic acid amide, pantothenic acid, riboflavin at 10 µg each/ml., vitamin B_{12} at 0.5 µg/ml. and biotin at 0.1 µg./ml.) and nucleic acid precursors (adenine, guanine, thymine, cytosine, thymidine and adenosine at 100 µg. each/ml.) were stored at 4^{o}. Aqueous solutions of amino acids (alanine, arginine, aspartic acid, cysteine and cystine, glutamic acid, histidine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan and tyrosine) at 10 mg. each/ml. were steam sterilised at 5 lbs. for 15 min. in Bijoux bottles and stored at 4^{o}. Stock aqueous solutions of tryptophan (0.5% w/v) and vitamin-free casamino acids (Difco Labs., Michigan, USA) (2.0% w/v) were sterilised at 15 lbs. for 30 min. and of cysteine (0.25% w/v) at 5 lbs. for 15 min.; all solutions were stored at 4^{o}.

b. Porphyrins and related compounds

Haemin (B.D.H. Ltd.) and protoporphyrin IX (B grade, Calbiochem. Ltd., Los Angeles, USA) were prepared as 0.1% (w/v) stock solutions according to Spencer and Herriot (1965) under aseptic conditions and stored at 4°. Solutions of δ-aminolaevulinic acid hydrochloride (ALA) (A grade, Calbiochem Ltd.) dissolved 0.1% (w/v) in water; cytochrome _c_ (A grade, Calbiochem Ltd.) dissolved 0.1% (w/v) in physiological saline; catalase (150,000 e.u./ml., B.D.H. Ltd.) diluted 1/10 (v/v) in physiological saline; and bovine albumin, fraction V (AFV) (Armour Pharmaceutical Co. Ltd., Eastbourne, England) dissolved 10% (w/v) in water, were each sterilised by Seitz filtration.

c. Miscellaneous

N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Aldrich Chemical Co., Inc., Milwaukee, Wisconsin, USA) was dissolved 0.1% (w/v) in sterile water and stored at 4°.

Deoxyribonuclease (DNAse) (B.D.H. Ltd.) was dissolved 0.1% (w/v) in sterile physiological saline and stored at 4°.

Streptomycin was supplied by Glaxo (Glaxo Laboratories Ltd., Greenford, Middlesex); Methicillin was supplied by Beecham (Beecham Research Laboratories, Brentford, England).

5. Isolation of bacterial mutants

a. Irradiation with ultraviolet light

Samples were taken from a culture of RI 2 during the exponential phase of growth in MD broth, centrifuged, resuspended in buffered saline and irradiated with a Fluorescence Lamp (Hanovia, Slough, Bucks., England) in an open, glass Petri dish (120 mm. diam.) approximately 15 cm. from the light source. The suspension was then diluted 1/10 (v/v) with MD broth and re-incubated for 2 hr. before 0.1 ml. samples were plated on MD agar containing 200 µg. streptomycin/ml.; pre- and post-irradiation dilutions were plated on MD agar to determine survival rate.

b. Exposure to copper

The method outlined by Weed (1963) was followed. Supplemented minimal medium (Spizizen, 1958), at a volume of 10 ml. in 100 ml. screw-capped bottles, was made $4x10^{-4}$M with respect to $CuSO_4$ and 25 µg./ml. with respect to haemin before inoculation with 1 ml. of an exponentially growing culture of strain RI 4 in MD broth, containing $1x10^8$ colony formers per ml. The bottles were incubated (static) at either 37^o or 43^o and samples were plated out on MD agar supplemented with 1 µg. haemin/ml., at intervals from the second to seventh day, for incubation at 37^o for 48 to 72 hr. The plates were then examined with a hand lens.

c. Exposure to N-methyl-N'nitro-N-nitrosoguanidine

The method of enrichment of auxotroph yield with penicillin described by Davis (1948) and Lederberg and Zinder (1948) was modified as follows in conjunction with brief exposure to MNNG. Bacteria of strain RI 4, growing exponentially in MD broth, were washed with buffered saline (pH 7) and resuspended in the same volume of buffered saline which was then inoculated to give a final concentration of 50 µg. MNNG/ml. Exposure for 30 min. at 37^o was terminated by immersion in melting ice before the tubes were centrifuged. The washed sediment was resuspended in 10 ml. MD broth supplemented with 5 µg. haemin/ml. and 500 µg. AFV/ml. and incubated overnight. Samples of 2 ml. each were added to 50 ml. minimal medium (GGM) supplemented with 200 µg./ml. of vitamin free cas-amino acids (Difco Labs., Michigan, USA) contained in a 250 ml. flask, which was then incubated for 4 hr. in the shaker before Methicillin (Beecham Labs.) was added to a final concentration of 1 mg./ml. After further overnight incubation, 0.1 ml. samples from a suspension in GGM of repeatedly washed bacteria were spread on GGM agar, supplemented for haemin auxotrophs (II, C.2.a.).

6. Bacterial transformation procedures

a. Preparation of DNA

Bacteria were harvested by centrifugation, in the late exponential phase of growth, of bulk aerated cultures (450 ml. TYE medium, supplemented for haemin auxotrophs if required, contained in 2 litre flasks). DNA was extracted according to Marmur (1961) and the concentration of the purified preparation was estimated by measurement of the optical density at 260 mμ in a Beckmann Spectrophotometer (Model DB, Beckmann Instruments Ltd., California, USA) (assuming $E^{1\ cm.}_{260\ m\mu} = 20$, with a solution of 1 mg. DNA/ml. Crude, deproteinised extracts were also made; their content of DNA was assessed chemically according to the method of Burton (1956).

b. Transformation procedures

The method of Anagnostopoulos and Spizizen (1961) was modified as follows. Bacteria were grown to a density of $5x10^8$ colony formers/ml.in growth medium (GM), then centrifuged, resuspended in 10 vol. transformation medium (TM) (see Table 2) and incubated in the shaker for 90 min. before DNA was added to a final concentration of 10 μg./ml. Exposure for 30 min. was terminated by the addition of DNAse to a final concentration of 100 μg./ml. and incubation was continued for a further 15 min. before 0.1 ml. samples of dilutions in minimal medium were spread on appropriately supplemented minimal agar for selection of required transformants. Colony counts were made on plates after 48 hr. incubation.

C. Results

1. Isolation of haemin auxotrophs

a. Irradiation with ultraviolet light

In the first experiment, in which there was 5% survival, the colonies which developed on the streptomycin plates after 48 hr. incubation were of two types; (a) creamy colonies more than 3 mm. in diameter, and (b) round, transparent colonies less than 0.5 mm. in diameter. The former greatly predominated.

Strain RI 4 was taken from a colony of the first type and was selected because of its colonial appearance, which was moist, domed and regular in comparison to the dry, flat, irregular colony of the parent strain (RI 2). It was resistant to 500 μg. streptomycin/ml. but was auxotrophic for tryptophan alone. The distinctive colonial appearance and the high level resistance to streptomycin were also demonstrated in the haemin auxotrophs (RI 6-11) derived from this strain, thus serving as additional markers.

Strain RI 5 was taken from a colony of the second type; it showed an increased size of colony formation on transfer to media containing blood and was resistant to 50 μg. streptomycin/ml. Colony formation by this strain on MD with and without added haemin is shown in Fig. 7. Identity as a haemin auxotroph was not established until a satisfactory minimal medium had been defined.

In two further experiments, in which the survival rate was less than 1%, small colony formers were encountered but none showed enhanced growth on haemin supplemented MD agar.

b. Exposure to copper (i) at 37°, (ii) at 43°

(i) Examination at 48 hr. of plates spread with material taken from flasks after seven days' incubation at 37° showed very occasional minute (less than 1 mm. diam.), transparent colonies interspersed between the parent-type (RI 4) colonies. One of these minute colonies gave rise to considerably larger colonies on BM, and was subsequently shown to be a haemin auxotroph. It was designated RI 6.

Figure 7. Colony formation by mutant strain, RI 5.

(a) Colony formation from diluted bacterial suspension of the strain on MD agar, after 48 hr.

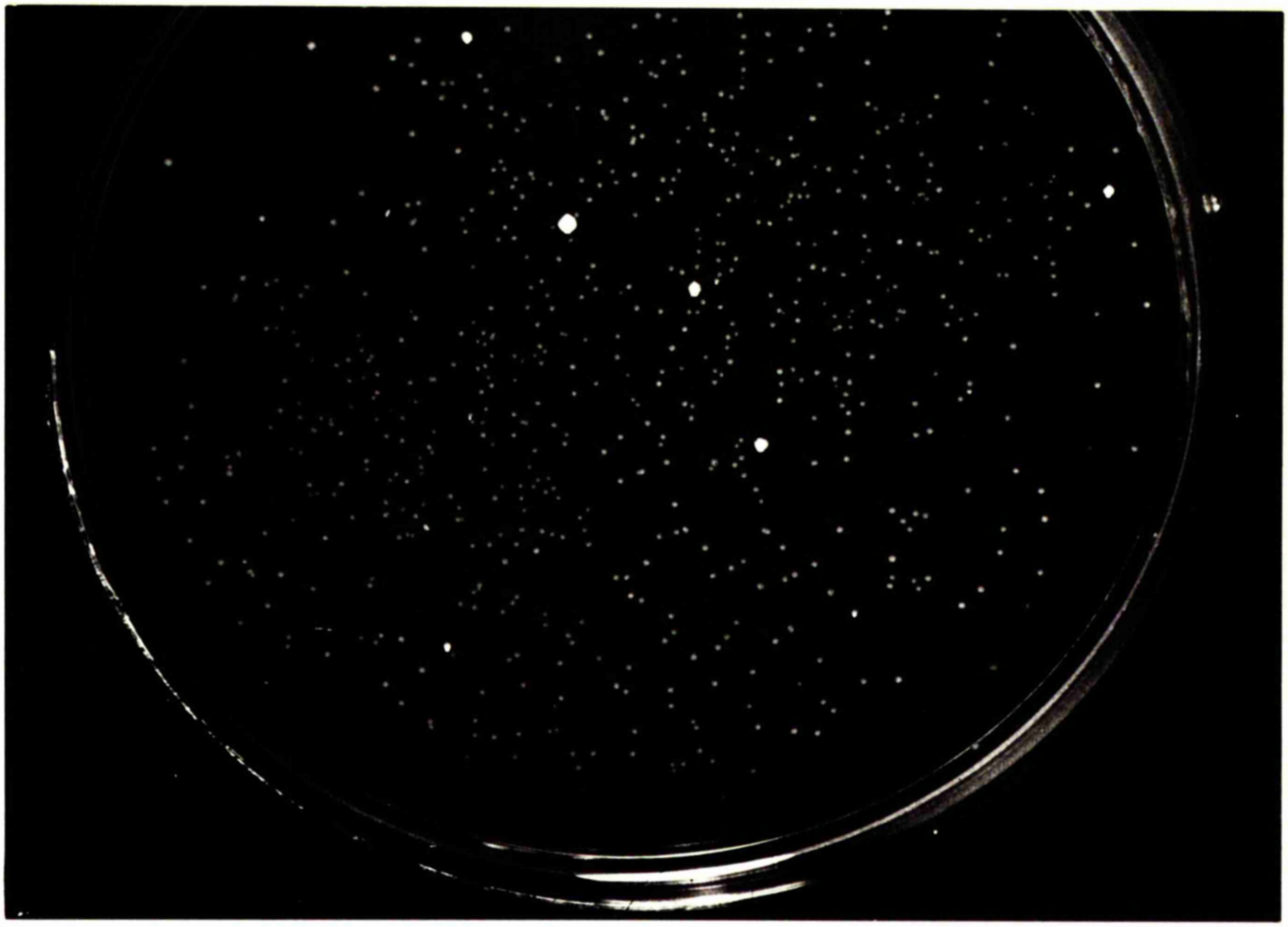
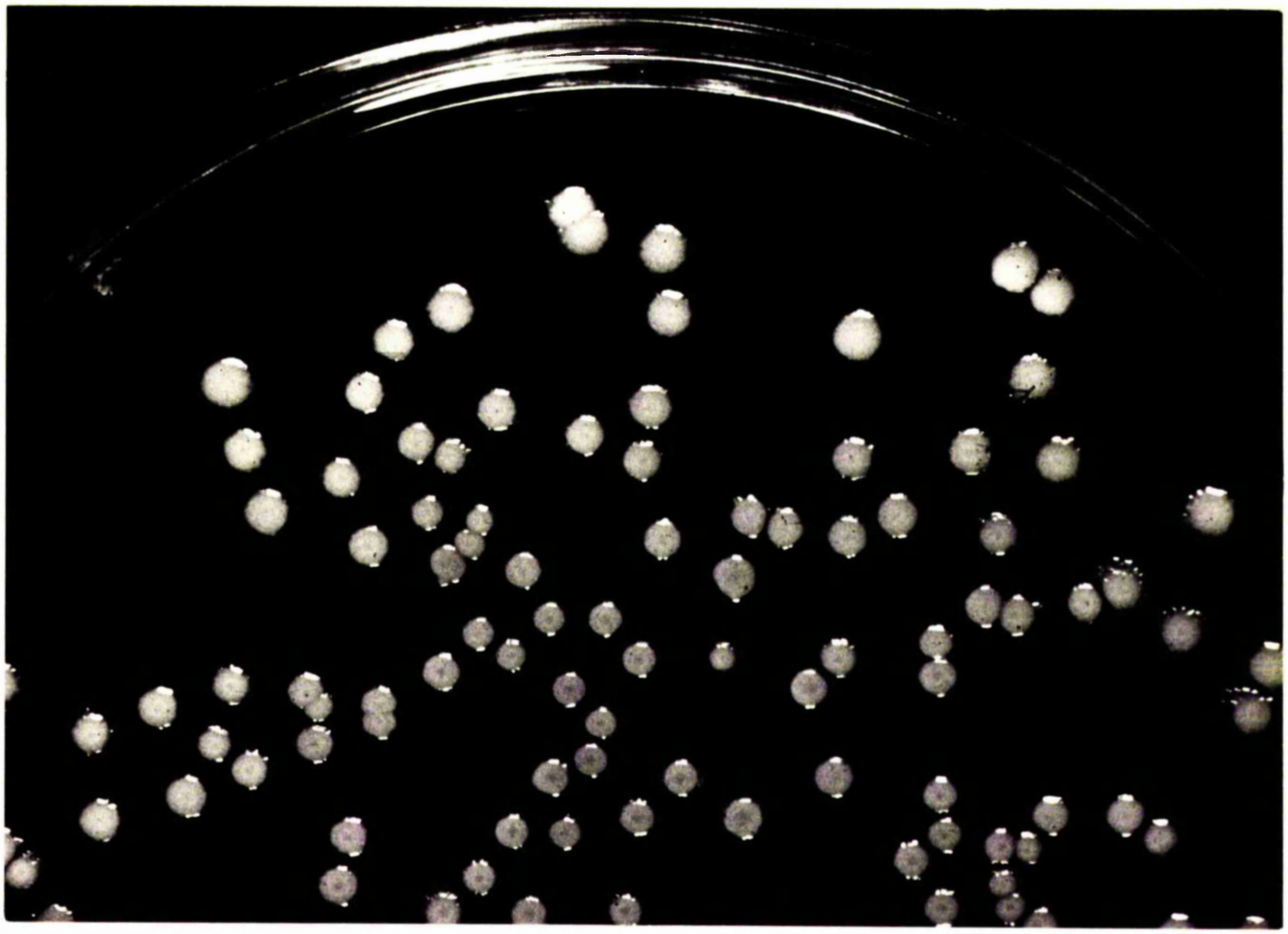


Figure 7. Colony formation by mutant strain, RI 5.

(b) Colony formation from diluted bacterial suspension (as Figure 7a) of the strain on MD agar supplemented with 5 µg. haemin/ml., after 48 hr.



No such appearances were found on plates inoculated from the flask at other times.

(ii) Minute colonies similar to those observed above were detected on plates, after 48 hr. incubation at 37°, which had been spread with material from the flasks incubated at 43°. The 24 hr. sample provided several examples but only one gave rise to larger colonies on BM. This strain, designated RI 7, was subcultured for maintenance with stock cultures. The 72 hr. sample gave only four minute colonies, three of which gave improved growth on BM. Subcultures of these three strains were taken and designated RI 8, 9 and 10, for maintenance with the other cultures.

These isolates were later confirmed as haemin auxotrophs on supplemented minimal media. Unfortunately, the method of initial plating did not permit assessment of the frequency of this mutational event.

c. Exposure to N-methyl-N'-nitro-N-nitrosoguanidine

In the first experiment, with greater than 99% killing from exposure to 100 μg. MNNG/ml., the colonies which developed had a normal appearance on MD agar, with and without haemin, and were not further examined. One hundred and sixty colonies, from an experiment in which an enrichment technique (II, B.5.c.) was used, were replica plated on minimal medium (GGM) with and without supplements for haemin auxotrophs. Only one colony satisfied the conditions of haemin dependence. The requirement for haemin was not absolute, however, as growth, though severely restricted, was observed on minimal medium without haemin. Such mutants are known as "leaky" (Braun, 1965a); mutations leading to quantitative deficiency of a specific enzyme, or a qualitative alteration that produces lower enzyme efficiency are believed to account for the phenomenon.

This mutant was designated RI 11, and a subculture introduced for maintenance with stock cultures.

2. Cultural characteristics of haemin auxotrophs

a. Definition of a minimal defined medium for haemin auxotrophs

The basal minimal medium for these studies comprised GGM supplemented with tryptophan, at 50 µg./ml. Addition of haemin (1-5 µg./ml.) to this basal medium was insufficient to support growth even with further supplements of mixtures of vitamins, nucleic acid precursors and casamino acids. However, a limited degree of colony formation was observed when the medium contained 0.5% (w/v) charcoal as well as haemin (5 µg./ml.) and casamino acids (200 µg./ml.).

The essential amino acids in the mixture were defined by observing the growth around "wells", charged with individual amino acid solutions (10 mg./ml.), cut in appropriately supplemented GGM agar. Only cysteine, or cystine, satisfied the requirement, and a concentration of 10-25 µg./ml. was later found to be sufficient. The more soluble cysteine was always used in subsequent experiments. Other SH containing compounds, methionine and thioglycollic acid (up to 250 µg./ml.) were incapable of replacing cysteine. These results are summarised in Table 3.

The ability of other substances to replace charcoal in the medium is evident from the results of experiments shown in Table 4. Good results were consistently obtained with bovine albumin, fraction V (AFV). At standard concentrations of cysteine (10-25 µg./ml.) a supplement of 100-500 µg. AFV/ml. promoted optimum colony formation (see Fig. 8). It was also observed, however, that at high concentrations of cysteine (500 µg./ml.) supplements other than tryptophan could be omitted, although resultant colony formation was sub-optimal.

Haemin auxotrophs of <u>Bacillus subtilis</u> (directly or indirectly derived from RI 2) were routinely cultured on a chemically defined minimal medium composed of GGM, with the following supplements (in µg./ml.), tryptophan (50), cysteine (10), AFV (500) and haemin (5). Colony formation after 48 hr. was almost equivalent to that obtained on BM after 24 hr.

Requirements in fluid media were identical.

Table 3. Supplements to a minimal medium for growth of isolates RI 5-10.

Dilution of bacterial suspensions in minimal medium (GGM + 50 µg. tryptophan/ml.) were plated on solid minimal medium supplemented as shown, at quantities given in text, and incubated 48 hr. at 37°.

haemin	vitamins and nucleic acid precursors	amino acids	charcoal	cysteine	Strain RI 5-10 Colony formation at 48 hr.
present	-	-	-	-	no growth
present	present	present	present	-	no growth
present	-	present	present	-	all distinct >2 mm. diam.
present	-	-	present	present	all distinct >2 mm. diam.
-	present	present	present	-	no growth

Table 4. Definition of optimum supplements to a minimal medium for growth of isolates RI 5-10.

Dilutions of bacterial suspensions in minimal medium (GGM) were plated on solid medium (GGM + 50 μg. tryptophan/ml. + 5 μg. haemin/ml.) supplemented in quantities stated in text, unless indicated otherwise, as shown in the table.

Cysteine (µg./ml.)	charcoal	AFV	crystalline albumin	DEAE* cellulose	DEAE* dextran	COM* cellulose	Strain RI 5-10 Colony formation at 48 hr.incubation
10	present	-	-	-	-	-	all distinct >2 mm. diam.
10	-	present	-	-	-	-	all distinct >2 mm. diam.
10	-	-	present	-	-	-	no growth
10	-	-	-	present	-	-	all distinct <2 mm. diam.
10	-	-	-	-	present	-	no growth
10	-	-	-	-	-	present	no growth
500	-	-	-	-	-	-	all distinct <2 mm. diam.

*DEAE = diethylaminoethyl, COM = carboxymethyl; each constituent added to make 0.25% w/v in medium.

Figure 8(a). Colony formation by strain RI 2 (S 168) on minimal medium.

An aliquot of a diluted bacterial suspension in minimal medium (GGM = gGM of Figure) was plated on agar medium supplemented as shown. The photograph was taken after 72 hr. incubation at 37^{o}. (magnification x 2 approx.)

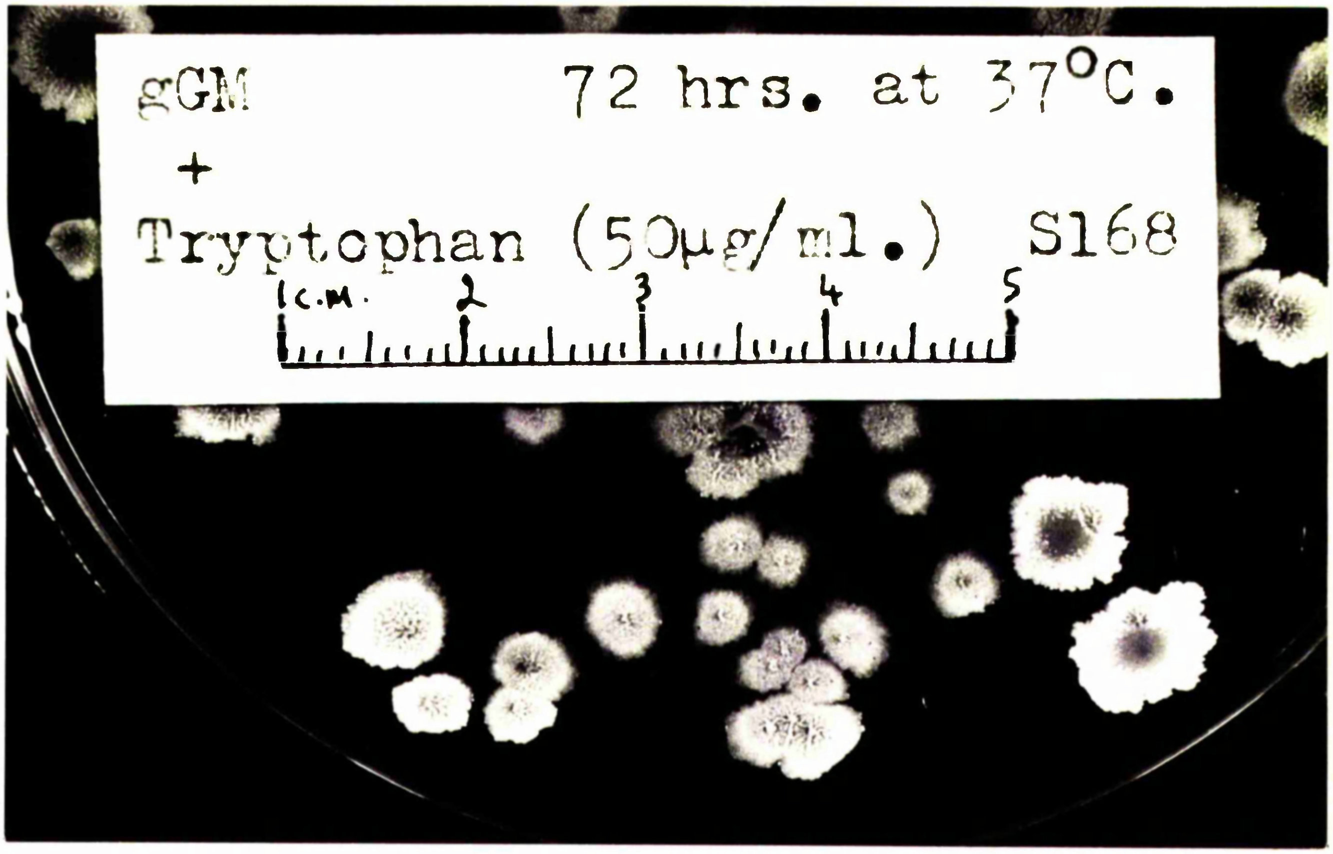

gGM 72 hrs. at 37°C.
+
Tryptophan (50µg/ml.) S168
1c.m. 2 3 4 5

Figure 8(b). Colony formation by strain RI 5(d_1) on minimal medium.

An aliquot of a diluted bacterial suspension in minimal medium (GGM = gGM of Figure) was plated on agar medium supplemented as shown. The photograph was taken after 48 hr. incubation at 37^{o}. (magnification x 2 approx.)

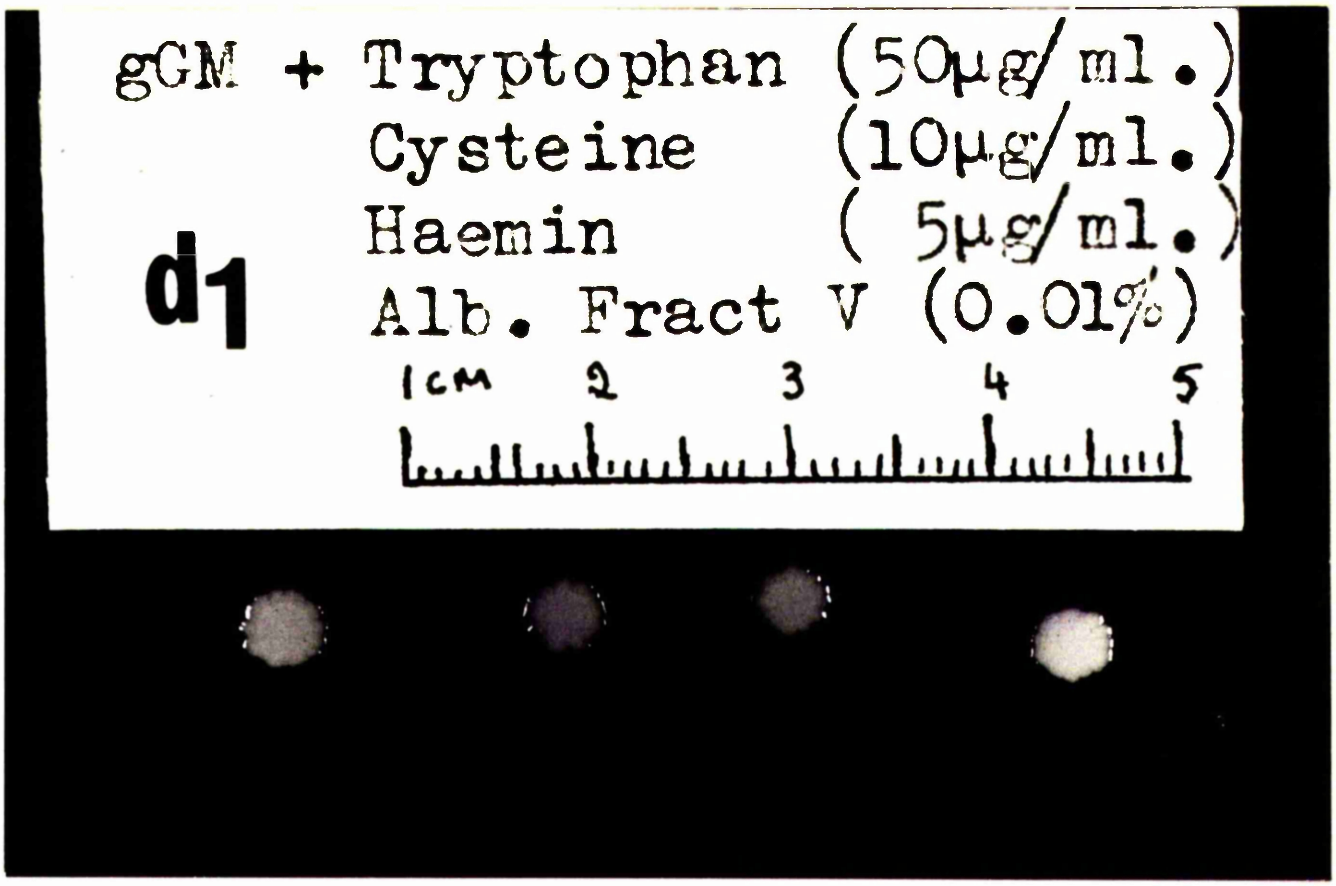
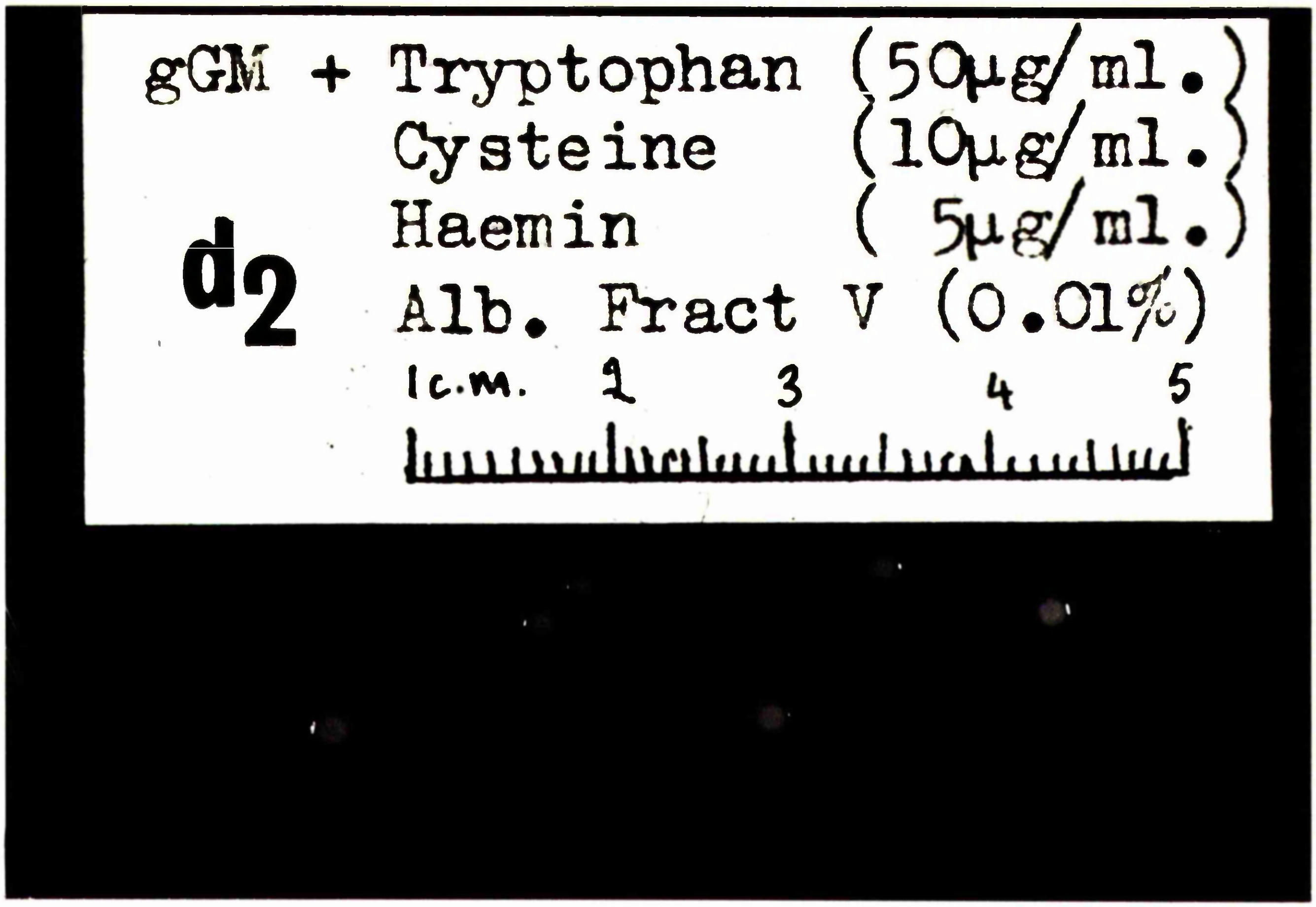

gGM + Tryptophan (50µg/ml.)
Cysteine (10µg/ml.)
Haemin (5µg/ml.)
Alb. Fract V (0.01%)
d1
1cm 2 3 4 5

Figure 8(c). Colony formation by strain RI 6 (d_2) on minimal medium.

An aliquot of a diluted bacterial suspension in minimal medium (GGM = gGM of Figure) was plated on agar medium supplemented as shown. The photograph was taken after 48 hr. incubation at 37^{o}. (Magnification x 2 approx.)


gGM + Tryptophan (50µg/ml.)
Cysteine (10µg/ml.)
Haemin (5µg/ml.)
Alb. Fract V (0.01%)
d2
1c.m. 2 3 4 5

b. Porphyrin utilisation and synthesis by auxotrophs.

(i) Haemin concentration range. The range of haemin concentration necessary to support growth showed an upper and lower limit. Whilst 2.5-5 μg./ml. promoted adequate colony formation on solid, defined media, concentrations between 1 and 2 μg./ml. were satisfactory in fluid media, although a uniform bacterial density was not reached in every experiment. Quantities in excess of 5 μg./ml. proved inhibitory to growth in both solid and fluid media.

Concentrations below 1μg./ml. failed to support growth (see Table 5).

Catalase, which destroys hydrogen peroxide, allowed a considerable reduction in the quantity of haemin required for optimal growth of the haemin auxotrophs. This "sparing" effect was observed in solid and fluid media; with 40 e.u. catalase/ml. a concentration of 0.1 μg. haemin/ml. was sufficient for optimal growth (see Fig. 9).

(ii) Precursor utilisation. None of the mutants grew when haemin was substituted by protoporphyrin IX, or the porphyrin containing compounds, cytochrome c and catalase. Coproporphyrin, uroporphyrin and porphobilinogen were not tested for their ability to replace haemin. However, two isolates, RI 5 and RI 6, were capable of utilising δ-aminolaevulinic acid (ALA), the first specific intermediate in the pathway to porphyrin synthesis (see Fig.10), and grew well in minimal medium supplemented only with tryptophan and 2.5 μg. ALA/ml.; no growth was obtained when ALA was substituted by pyridoxal phosphate (10^{-5}M), which is known to be a cofactor for the enzyme ALA synthetase (Lascelles, 1964). The growth response of RI 5 to ALA in minimal medium is illustrated in Fig. 11.

c. Generation time of bacterial strains. The mean generation time of strain RI 1 (wild type) in MD, and in minimal medium used for transformation experiments, was 46 min.

haemin (µg./ml. ⟶)		5	2.5	1	0.5	0.1	0.05	0
with catalase	RI 5-10	+++	+++	+++	+++	+++	±	-
	RI 11	-	+++	+++	+++	+	±	±
without catalase	RI 5-10	+++	+++	++	-	-	-	-
	RI 11	+++	+++	+++	+	+	±	±

Table 5. Growth response of isolates RI 5-11 to haemin with and without catalase (40 e.u./ml.)

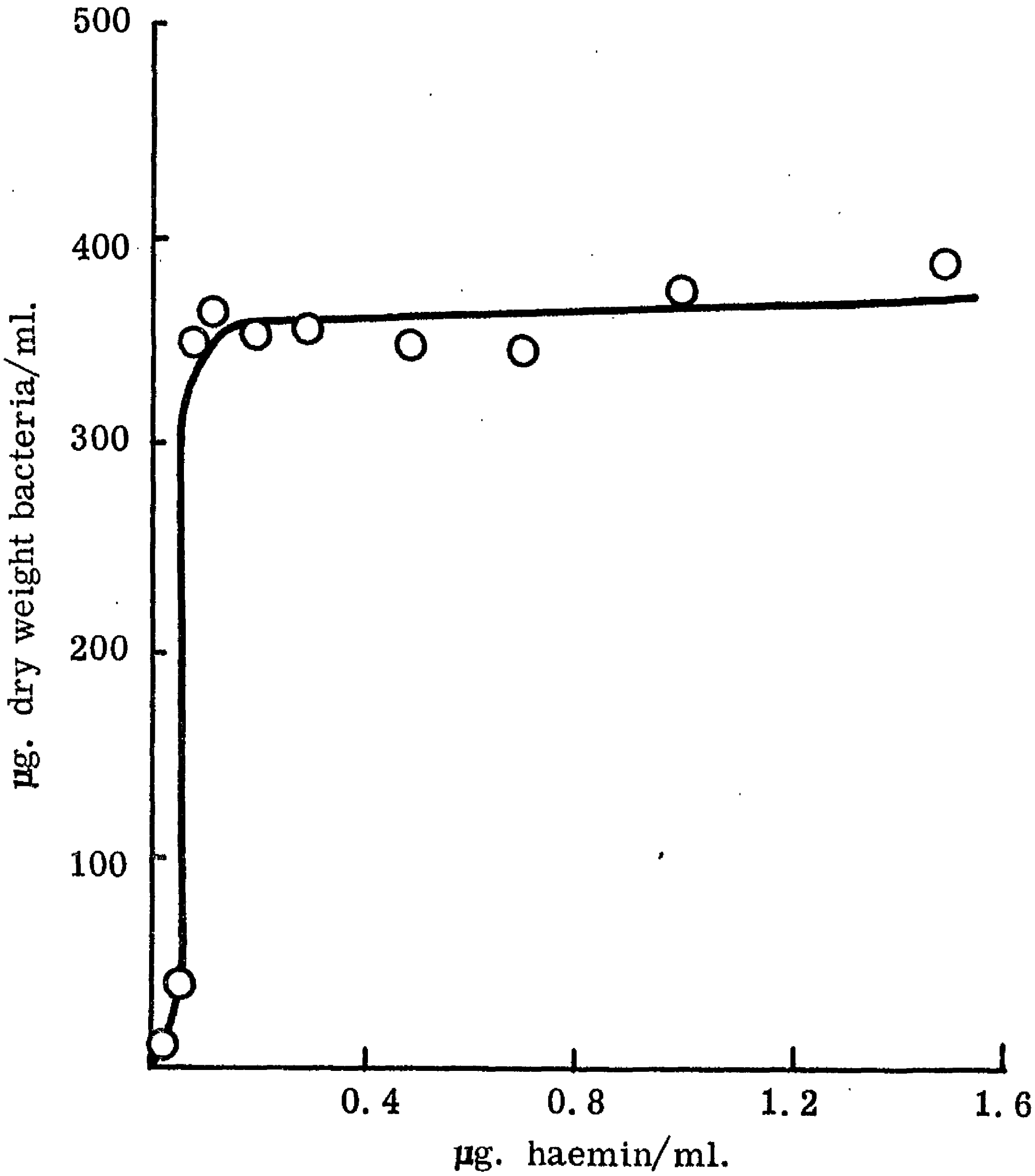
Dilutions of bacterial suspensions in minimal medium (GGM) were plated on solid medium (GGM + 50 µg. tryptophan/ml. + 10 µg. cysteine/ml. + 500 µg. AFV/ml.) supplemented with haemin and catalase as indicated. Colony formation after 48 hr. at 37° was noted as:

+++ = >2 mm. diam.
++ = 1-2 mm. diam.
+ = <1 mm. diam.
± = pin-point "dust" colonies.
\- = no growth.

The results demonstrate the "sparing" effect of catalase for haemin and confirm the "leaky" nature of mutant RI 11.

Figure 9. Growth response of strain RI 5 to haemin with catalase.

Aliquots of 10 ml. adequately supplemented minimal medium, with the stated concentration of haemin, plus catalase (40 e.u./ml.), in 100 ml. flasks were inoculated with 5×10^4 washed colony formers from an overnight culture of the strain in supplemented minimal medium containing 2 µg. haemin/ml. and catalase (40 e.u./ml.). The optical density value after 22 hr. shaking incubation was converted to dry weight of bacteria per ml. The bacterial yield is plotted as a function of the initial concentration of haemin in the medium.


500
400
300
200
100
µg. dry weight bacteria/ml.
0.4
0.8
1.2
1.6
µg. haemin/ml.

succinyl-CoA + glycine

↓

δ-aminolaevulinic acid

↓

porphobilinogen

↓

uroporphyrinogen

↓

coproporphyrinogen

↓

protoporphyrin

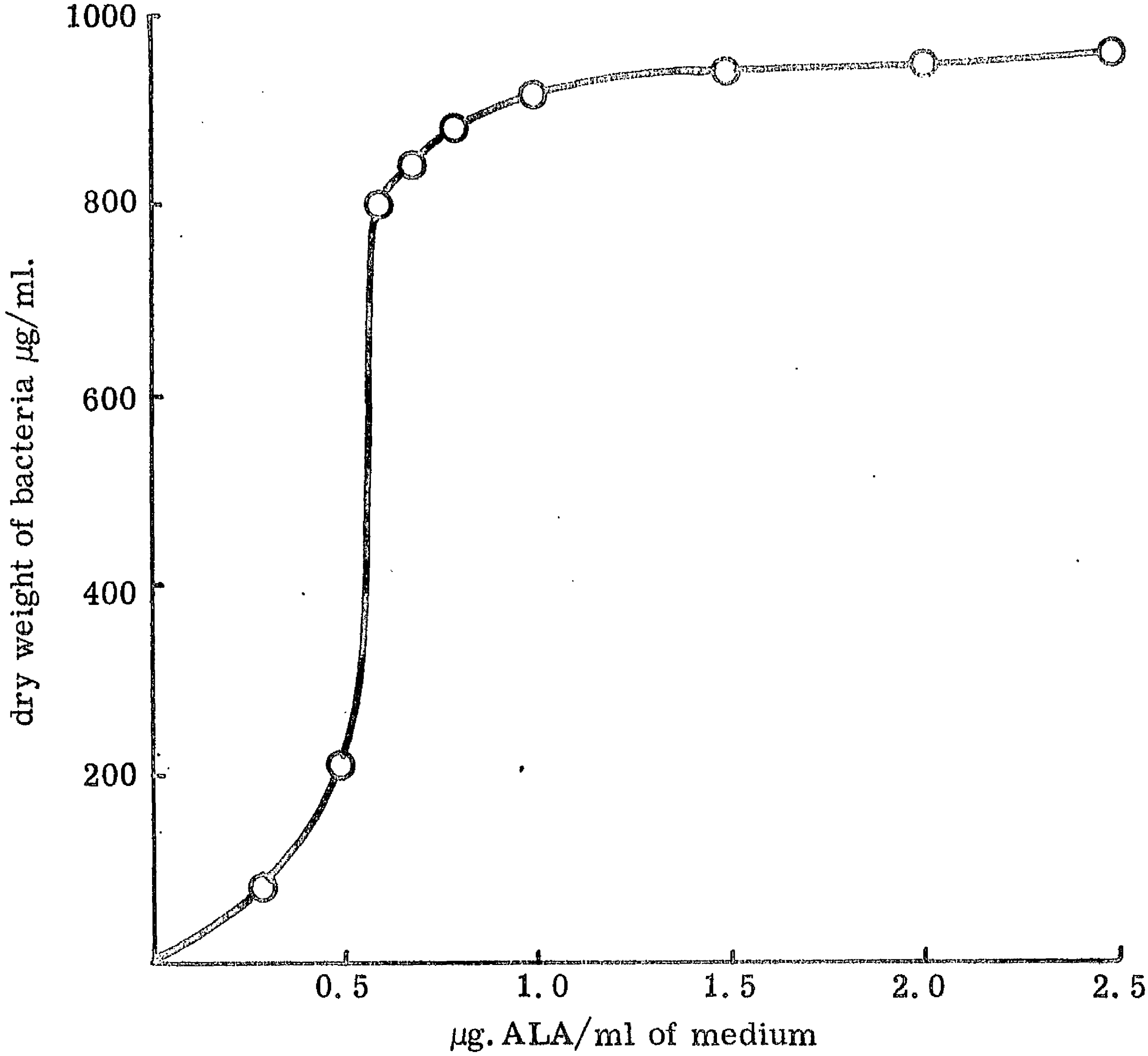
↓ Fe^{2+}

haem

Figure 10. Pathway of porphyrin synthesis. (from Lascelles, 1964).

Figure 11. Growth responses of strain RI 5 to δ-aminolaevulinic acid.

Aliquots of 10 ml. supplemented minimal medium in 100 ml. flasks were inoculated with 5 x 10^4 washed colony formers from an overnight culture of the strain in supplemented minimal medium containing 2.5 µg. ALA/ml. The optical density value after 20 hr. incubation was converted to dry weight of bacteria per ml. The bacterial yield is plotted as a function of the concentration of ALA.


1000
800
600
400
200
dry weight of bacteria µg/ml.
0.5
1.0
1.5
2.0
2.5
µg. ALA/ml of medium

The mean generation time of strains RI 5 and 6 in ALA supplemented minimal medium was 70 and 65 min., respectively. The generation time of the other mutants in haemin-supplemented media was not examined.

d. Morphology: cells and colonies. The cellular morphology and reaction to Gram stain was identical in the parent strains and the mutants derived from them. The colony formed by haemin auxotrophs on supplemented minimal medium after 48 hr. incubation was small, 2-3 mm. in diameter, moist and more regular (Fig. 8a) in comparison to the 5 mm. diameter, dry, irregular colony of strain RI 2 (Fig. 8c), from which they were all derived. This difference was not further examined.

3. Transformation of haemin auxotrophs.

Modification to the method of Anagnostopoulos and Spizizen (1961) was required in order to increase the yield of transformants to acceptable levels. The yield of transformants to haemin independence obtained by exposure of RI 5 to DNA extracted from RI 1 (wild type), in growth and transformation media supplemented with haemin, was influenced by the cell density at time of transfer from GM to TM, as shown in Table 6. Table 7 shows the increase in the number of transformants when cells are transferred from GM at a cell density of 5×10^{8} colony formers/ml. and the period of incubation in TM is increased, prior to exposure to DNA for 15 min. The procedure routinely adopted and already outlined (II,B.6.b.) combined the most optimal conditions. The DNA was always used at saturation level (10 µg./ml.) (see Table 8).

The results of transformation experiments with the mutants are summarised in Table 9.

Transformation rates of the order of 0.1%, acceptable for B. subtilis strains, were regularly obtained with the strains RI 5 and RI 6 when the experiments were performed in media supplemented with ALA. In haemin supplemented media with the same strains the yield of transformants was reduced by a factor of 10 (0.01%), even with reduced concentrations (1 µg./ml.) of haemin in the presence of catalase (40 e.u./ml.). Strain RI 11 was transformed to haemin independence in haemin media at a

Transformation of RI 5 to tryptophan independence, in medium supplemented with haemin, by DNA from RI 1

Table 6. The effect of increased cell density in growth medium (GM) before transfer to transformation medium (TM) on the recovery of transformants.

Samples were taken from a culture of the strain in GM, at the stated cell density. Transformation experiments were performed as described in text; cells were exposed to DNA for 15 min. after 90 min. in TM.

Table 7. The effect of increased incubation time in TM on the recovery of transformants.

Transformation experiments were performed as described in the text. Cells were transferred to TM at a density of 5×10^{8} cells/ml. in GM and exposed to DNA for 15 min., after incubation for the stated times.

Cell density in GM ($\times 10^{-8}$/ml.)	Transformants to haemin independence in 10^8 recipients
1.5	1×10^2
2.2	3×10^4
5.0	7×10^4

Incubation time in TM (min at 37^o)	Transformants to haemin independence in 10^8 recipients
0	6×10^2
60	2.3×10^3
90	1.7×10^4
120	5.0×10^4

Transformation of RI 5 to tryptophan independence, in medium supplemented with haemin, by DNA from RI 1.

Table 8. The optimum DNA concentration for recovery of transformants.

Transformation experiments were performed as described in the text. Cells were transferred to TM at a density of 5×10^8 cells/ml. in GM and exposed to DNA at the stated concentration for 30 min. after incubation in TM for 90 min.

DNA concentration in TM (μg./ml.)	Transformants to haemin independence in 10^8 recipients
10	7×10^4
2.5	4×10^4
1.0	3×10^4
0.1	4×10^4
0.01	5×10^3
0.005	7×10^3

Table 9. Transformation of Bacillus subtilis strains.

Transformation experiments were performed as described in the text. Samples of 0.1 ml. from appropriate dilutions were plated on suitably supplemented minimal medium. Averages of colony counts on three plates after 48 hr. incubation are expressed as transformants per 10^8 recipients.

Recipient strain	Donor strain (DNA conc. 10 µg./ml.)	Transformation to independence in 10^8 recipients				
		Trp	Hem	Trp + Hem	His	Trp + His
RI 2	RI 1	1.5×10^5	.	.	.	.
RI 4	RI 1	1.0×10^5	.	.	.	.
RI 5*	RI 1	1.4×10^5	1.3×10^5	4.0×10^3	.	.
RI 5*	RI 6*	< 10	< 10	< 10	.	.
RI 5*	RI 9	< 10	1.0×10^5	< 10	.	.
RI 6*	RI 1	.	1.0×10^4	.	.	.
RI 7	RI 1	.	5.0×10^2	.	.	.
RI 8	RI 1	.	1.5×10^2	.	.	.
RI 11	RI 1	.	2.0×10^4	.	.	.
RI 3	RI 1	3.0×10^4	.	.	2.4×10^4	2.4×10^4
RI 3	RI 7	.	.	.	1.0×10^4	.

Trp = tryptophan, Hem = haemin, His = histidine, (*) = utilizes ALA rate of 0.2%, but the other mutants (RI 7, 8, 9, 10) failed to give sufficient transformants for any marker (0.0005% or less). AFV has been reported to inhibit transformation (Spizizen, 1958). That this poor rate of transformation was not due to the AFV, an essential supplement in haemin media, was shown by the success of transformation of RI 5 to haemin independence (0.05%) in ALA media supplemented with AFV.

D. Discussion.

1. Haemin auxotrophs

A streptomycin resistant mutant of *Staphylococcus aureus* was reported by Jensen and Thofern (1953) to show a requirement for haemin. Beljanski and Beljanski (1957) isolated a similar mutant from *Escherichia coli* by a process of selection using streptomycin. The isolation, in the present study, of a haemin auxotroph of *Bacillus subtilis* on a medium containing streptomycin provides another example of the unexplained relationship between streptomycin and the induction of haemin auxotrophy. Streptomycin is believed to induce errors in translation of mRNA (Davies, Gilbert and Gorini, 1964). To what extent this action may have contributed to, or complemented, the mutagenic effect of UV irradiation is not known.

Mutants of *Escherichia coli* have been isolated by Weed (1963) following prolonged exposure to copper. The examples of mutation (RI 6, 7, 8, 9 and 10) induced with copper in this study provide further examples of the extraordinary effects of exposure to this metal. From an examination of the heat denaturability of DNA treated with copper, Eichorn (1962) concludes that an interference with the hydrogen bonds between strands of the double helix of DNA could be responsible for profound effects upon its secondary structure. It is possible that mutation is induced in the present study by errors of hydrogen bonded pairing between nucleic acid bases on DNA replication.

The mutagenic properties of MNNG are first reported by Mandell and Greenberg (1960). The actions of this chemical will be discussed elsewhere (III, A.1., 2., and D.3.iv.).

It is significant that auxotrophy for haemin can be induced by a variety of agents. Whilst it is regretted that a numerical assessment of the frequency of the mutation was not, or could not be, made, repetition of the experiments, with MNNG for example, would be expected to provide such information. Wulff (1967) in an examination of 1,500 colonies of survivors after MNNG treatment found one mutant *Escherichia coli* with a growth requirement for ALA.

2. Chemically defined growth medium for haemin auxotrophs

The definition of supplements to a minimal medium which allow full aerobic growth of haemin auxotrophs of Bacillus subtilis is an improvement to the peptone-glucose medium used by Jensen and Thofern (1953) and Beljanski and Beljanski (1957). The minimal concentration of haemin (0.1 µg./ml.) to which the response of the strains is optimal, in the presence of catalase, compares favourably with the minimal porphyrin requirement of Haemophilus influenzae strains (Granick and Gilder, 1946) and confirms the specific nature of the dependence.

The function of cysteine and AFV in the medium is not known. The ability of ALA utilising strains (Anderson and Ivanovics, 1967; Wulff, 1967) to grow in minimal media supplemented with ALA alone indicates the association of cysteine and AFV with haemin-containing media only. It is most probable that the function of cysteine is to control the oxilation-reduction potential of the medium in order to maintain the porphyrin molecule in the reduced state, readily assimilable to the organism for incorporation into porphyrin compounds (Lascelles, 1964).

Davis and Dubos (1947) find AFV to be most effective in promoting growth in the presence of inhibitors, such as unsaturated fatty acids, and attributed this property to the capacity of the molecule to bind, and slowly release, substances inhibitory to growth at higher concentrations. Haemin is growth-limiting above certain concentrations (II, C.2.b.i.); it is possible that AFV maintains the concentration at non-toxic levels. A similar explanation may account for the ability of cationic cellulose (DEAE) to replace AFV, but does not explain the ability of high concentrations (500 µg./ml.) of cysteine to replace AFV in the minimal medium (Table 4).

The activity of catalase in sparing haemin is explained by the known destructive effect of H_2O_2, the substrate for catalase, on haem compounds (Gilder and Granick, 1947).

3. Porphyrin precursor utilisation by haemin auxotrophs

The strains RI 5 and 6 utilise ALA and, therefore, possess the enzymes required for porphyrin synthesis, with the exception of ALA synthetase. This enzyme catalyses the condensation of succinyl-coenzyme A with glycine to form δ-aminolaevulinic acid (Lascelles, 1964). Whether this deficiency is quantitative or qualitative is not known, but it does not appear to be due to lack of cofactor, pyridoxal phosphate (II, C.2.b.ii). The inability of RI 5 and 6 to utilise protoporphyrin IX does not, therefore, result from a deficiency of the enzyme "ferro-chelatase", which catalyses the insertion of iron into protoporphyrin. The mutant strain of Jensen and Thofern (1953) is described as deficient in this enzyme because of an inability to utilise protoporphyrin.

The blockage in the pathway of porphyrin synthesis in the mutants RI 7, 8, 9, 10 and 11 was not determined.

Interest in the synthesis of porphyrins has been stimulated by the results of Tschudy _et al_. (1965), who demonstrate an elevated level of ALA synthetase in the liver of a patient with acute intermittent porphyria. It is now believed (Kappas and Granick, 1968) that this enzyme plays the key rôle in the regulation of porphyrin synthesis in mammalian cells. Bacterial strains, such as the isolates RI 5 and 6, with chromosomal mutations in the gene unit (operon) which determines the activity of this enzyme might provide a valuable experimental system to be used, in studies of genetic recombination, to obtain information about genetic factors controlling porphyrin synthesis in cells.

4. _Transformation experiments_

The purpose of this preliminary study is to demonstrate that a detailed analysis with a larger number of mutants might contribute to the understanding of the genetic control of porphyrin synthesis in _Bacillus subtilis_. This work would serve as a useful hypothesis for the study of mammalian work.

The concept of the bacterial chromosome as a linear sequence of units arranged in a closed circle is explained previously (II, A.). Strain RI 3 (genotype _trp_, _his_) gives a high rate of transformation to independence for both histidine and tryptophan which is equivalent to the rate for either alone (see Table 9). This result is confirmation of the linkage, or proximity to each other, of the gene loci for histidine and tryptophan synthesis in this strain (Nestor, Schafer and Lederberg, 1963). In comparison, the low recovery of double transformants, to haemin and tryptophan independence, from RI 5 treated under similar conditions with DNA from the same source, RI 1, indicates that the hem and trp loci are distant from each other on the chromosome.

Wulff (1967), in a similar analysis of an Escherichia coli mutant auxotrophic for ALA, finds that the mutation site probably lies in the region pro-thr-leu of the E. coli chromosome. This region is distant from the trp locus (Braun, 1965b).

Tomasz (1966) and Thorne and Stull (1966) stress the importance of bacterial transfer from GM to TM at an optimum cell density if a high yield of transformants is to be obtained. The requirement for incubation beyond standard times in GM or TM for the haemin auxotrophs is probably related to the increased generation time of these strains (II, C.2.c). However, an intrinsic low level of competence in the strains, RI 7-10, is believed to account for the poor efficiency of transformation. It is also possible that these isolates are siblings and possess the same genotype. This conclusion follows from the consideration of the method of isolation from broth culture (II, C.1.b.)

E. Conclusions

1. Seven Bacillus subtilis mutants with a nutritional dependence for haemin are isolated, after irradiation with UV (one), exposure to copper (five), and treatment with N-methyl-N'-nitro-N-nitrosoguanidine (one).

2. A chemical, minimal medium is defined which optimally supports the growth of these mutants.

3. Two isolates are demonstrated to carry mutations in the gene locus determining the enzyme δ-aminolaevulinic acid synthetase, which catalyses the first reaction in the synthesis of porphyrins.

4. Bacterial transformation experiments are carried out with satisfactory efficiency in three isolates, Rf 5, 6 and 11. The results demonstrate that the hem locus is not proximal to the trp locus on the Bacillus subtilis chromosome.

5. A detailed analysis of bacterial transformation in a greater number of competent haemin auxotrophs may provide valuable information about the units of the gene operon determining porphyrin synthesis.

The report of activity of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in animals (Schoental, 1966b) prompted the continuation of this work as an examination into the effects of MNNG on mammalian cell systems. These studies are presented in Part III of this thesis.

PART III

STUDIES WITH MAMMALIAN CELLS

III. STUDIES WITH MAMMALIAN CELLS

A. Introduction

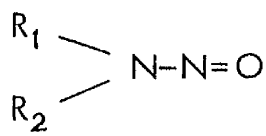
1. Chemistry of N-methyl-N'-nitro-N-nitrosoguanidine

The synthesis of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Fig. 12) was reported in 1947 (McKay and Wright, 1947) and this was followed by an account of its suitability for the preparation of diazomethane, as a methylating agent (McKay, 1948). The only disadvantage to handling MNNG was noted to be a skin irritation with vesicant reaction. In view of an earlier report by Davis and Rosenquist (1937) on the reaction of nitrosoguanidines with aliphatic amines, MNNG also would be expected to react with amines. This was indeed reported by McKay and Wright (1947), Henry (1950) and McKay (1949, 1952). Henry (1950), who commented on the formation of methylaniline from aniline reacted with MNNG, did not believe that diazomethane was the active agent derived from MNNG, and instead proposed monomethylnitrosamine. However, monomethylnitrosamine is known to be unstable (Müller, Haiss and Rundel, 1960) and might be expected to decompose to either diazomethane, or carbonium ion, depending on the pH.

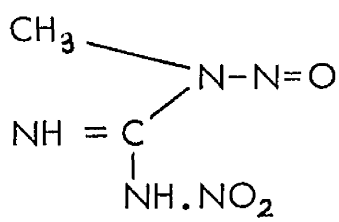
The stability of MNNG is greatly influenced by pH, the maximum stability being around pH 5 with decomposition under more acid conditions to yield nitrous acid, but under alkaline conditions diazomethane is produced (McKay and Wright, 1947). The instability of MNNG, and other N-methyl-N-nitroso compounds under different conditions of pH, and also of illumination, is commented upon by McCalla, Reuvers and Kitai (1968). Sensitivity to ultraviolet light (UV) is a feature of nitrosamines (Magee and Barnes, 1967). Indeed Preussmann, Daiber and Hengy (1964) utilise the transfer of the nitroso group to a suitable acceptor group, after irradiation with UV light, for the detection of nitrosamines after chromatography.

The solubility of MNNG in aqueous solvents is limited to approximately 0.04 M at 25° (Singer and Fraenkel-Conrat, 1967). It has been suggested that MNNG is lipophilic (Freese and Bautz-Freese, 1966).

Figure 12. Chemical structure of N-methyl-N'-nitro-N-nitrosoguanidine.



General formula for nitrosamines



N-methyl-N'-nitro-N-nitrosoguanidine

Moreover, the activity of a number of analogues of MNNG against intracerebral leukaemia in mice was attributed by Schabel et al. (1963) to their lipoid solubility, supportive chemical data being given.

2. Biological reactivity of N-methyl-N'-nitro-N-nitrosoguanidine

Initial interest in the nitrosoguanidines centred on the derivation from them of "flashless propellants" (see McKay, 1952). Biological activity of MNNG is known in a wide variety of cellular systems, ranging from viruses to intact mammals, and is best considered in groups, as follows

a. Bacteria and viruses

MNNG was proclaimed as a new chemical mutagen for bacteria by Mandell and Greenberg (1960). Although the mechanism of its action was not understood, mutagenicity by MNNG was further studied by Adelberg, Mandel and Chen (1965). These authors declared MNNG as the most potent mutagen, giving rise to as high as 50% yield of mutants, with altered nutritional requirements, in a population of *Escherichia coli* K 12 exposed to MNNG. The compound is now commonly used as a bacterial mutagen, for example, by Mandell, Woody and Greenberg (1961), Eisenstark, Eisenstark and van Sickle (1965), Anderson and Ivanovics (1967) and also by Wulff (1967).

The ability of MNNG to induce prophage in lysogenic strains of bacteria has been demonstrated (Marjai and Ivanovics, 1964; Allan and McCalla, 1966; Malke, 1967). The mechanism of prophage induction is not understood, but it has been proposed that the induction of a break in the polynucleotide strand of DNA may be a significant factor (Freese and Bautz-Freese, 1966). Certainly, the ability of MNNG, or a derivative, to react with nucleic acids is the subject of discussion in the report by Singer and Fraenkel-Conrat (1967) on the effects of MNNG on tobacco mosaic virus (TMV). These authors conclude that reaction of MNNG with guanyl residues of RNA is the predominant effect, but that the conformation of the RNA in the virus particle plays a large part in the determination of the sites of reaction.

Reaction of MNNG with DNA is also indicated by the inactivation of extracted DNA, as transforming agent for bacteria, after exposure to MNNG (Terawaki and Greenberg, 1965).

b. Unicellular organisms and plants

Mutagenic effects were observed among seeds of Arabidopsis thaliana exposed to MNNG (Müller and Gichner, 1964) and chromosomal aberrations were noted in Vicia faba meristems after exposure to MNNG at pH 4.2, although not at pH 8.9 (Gichner, Michaelis and Rieger, 1963). However, effects of MNNG on elements other than the chromosomes were recorded, for example, by Gillham (1965), who isolated a number of non-chromosomal mutants (i.e with uniparental inheritance) from Chlamydomonas rheinhardi strains treated with MNNG. Another aspect of MNNG extra-chromosomal activity is the ability to induce the heritable loss of chloroplasts, or "bleaching", in Euglena gracilis (McCalla, 1965, 1966, 1967a). These intracellular organelles (chloroplasts) are necessary for the conversion of radiant energy for use by the cell (Green and Goldberger, 1967). Likewise, "petite" mutants were induced in Saccharomyces cerevisiae with MNNG by Nordström (1967). Such mutants were earlier shown by Ephrussi (1953) to result from mutations in intracellular organelles, the mitochondria. Chromosomal mutations in S. cerevisiae have been studied by Marquardt, Zimmerman and Schwaier (1964).

c. Mammals: cancer chemotherapy and carcinogenesis

As early as 1959 the activity of MNNG against selected murine leukaemias was recorded by Leiter and Schneiderman (1959) and by Goldin, Venditti and Kline (1959). Similar reports of anti-tumour activity followed in 1960 (Greene and Greenberg, 1960) and led to the testing of analogues of MNNG for anti-leukaemic activity (Skinner et al., 1960; Schabel et al., 1963). Possibly because of the potency of the analogues, or its general toxicity, interest in MNNG as a chemotherapeutic agent was not sustained.

Contrary to the above experiences of cancer chemotherapy, recent reports on the biological activity of MNNG have emphasised its importance as an agent of carcinogenesis. Schoental (1966b) induced squamous carcinomas of the forestomach of rats by feeding them a total dose of 10-50 mg. MNNG, but failed to induce tumours with either N-methyl-N-nitroguanidine or N-ethyl-N'-nitro-N-nitrosoguanidine. Tumours were also induced with a single dose of MNNG.

Druckrey *et al*. (1966) induced sarcomas at the injection site of MNNG in rats, and contrasted this with the production of tumours in organs remote from the site of administration of dialkylnitrosamines. Tumours were also induced with MNNG in rats by Sugimura, Nagao and Okada (1966) and by Sugimura and Fujimura (1967).

These reports of the carcinogenic activity of MNNG are not altogether surprising in view of the well documented activity of nitrosamine compounds as carcinogens (Magee and Schoental, 1964; Magee and Barnes, 1967; Druckrey *et al*., 1967).

3. The cellular target molecule for reaction with N-methyl-N'-nitro-N-nitrosoguanidine

A large number of different compounds are contained within a cell, for example, polysaccharides, phospholipids and proteins, which might react with a biological agent. However, recent interest has centred mainly on reactivity with proteins or nucleic acids, because of the importance of these molecules in cellular function.

a. Protein

The molecules of protein are composed of one or more polypeptide chains, which in turn consist of polymers of amino acids linked together by their carboxyl and amino groups. The known reactivity of MNNG with amines (see III, A.1.) might favour reaction with not only free amino acids but also with the N-terminal group of polypeptide chains or with any amino groups of their side chains, as in the case of lysine, which is a diamino acid.

The amino acid cysteine was found by Greenberg and Morris (1961) to be ten times as effective as glycine in the protection of *Escherichia coli* against toxicity by MNNG. The authors considered as possible mechanisms of protection both the reduction of the nitroso group and direct interaction of MNNG with thiol groups of cysteine. Reaction with thiol groups would appear to be common to all nitrosamides (Magee and Barnes, 1967). The reaction of a nitrosamide, N-methyl-N-nitrosourethane (MNUE), with cysteine, was examined closely by Schoental and Rive (1965), who found the main products of reaction, at room temperature and neutral pH, to be S-methylcysteine and S-ethoxycarbonylcysteine. Schoental (1966a) further discussed the instability of these compounds in relation to their isomerisation between adjacent thiol and amino groups, and considered the possible consequences of this effect on the catalytic activity of enzymes. She also suggested that proximity of thiol and amino groups might influence reaction of MNUE with particular groups of nucleic acids.

This reactivity of MNNG and other nitrosamines with amino and thiol groups may have importance in determining susceptibility of some regions of the chromosome to reaction, in view of the close association with DNA, in the chromosome, of protein histones (Bonner and Huang, 1963, 1964). These proteins have a rich content of arginine (a diamino acid) and, in some fractions, cysteine (Butler, 1966). Histidyl residues of proteins would also be susceptible to reaction with "free radicals" derived from the breakdown of MNNG, in view of the nucleophilic nature of the imidazole ring of histidine.

Proteins also serve a function as structural components in a cell. Arcos and Arcos (1962), in an extensive review of chemical carcinogenic activity, argued that the biological activity of the nitrosamines might be related to their reaction with the structural proteins of membranes, and quoted examples of an altered physiological state of membranes induced by various hepatotoxic agents. Other proteins of importance in possible reaction with such agents would be genetic regulatory molecules (I, C.). Such a reaction, between carcinogen and repressor molecule, forms the basis of the hypothesis on the mechanism of carcinogenesis according to Pitot and Heidelberger (1963).

b. Nucleic acids

Evidence is given above (III, A, 2) of the activity of MNNG as a mutagen and inducer of chromosomal abnormalities. From the outline of the function of the nucleic acids as determinants of cell function already given it is pertinent to consider the possibilities of their reaction with MNNG.

The alkylation of nucleic acids by a variety of mutagens and carcinogens, among them, nitrosamines, has been reviewed by Lawley (1966). The consequences of alkylation on hydrogen bonded base pairing of nucleosides has been discussed already (II, A). The alkylation of adenine or guanine in DNA induces instability of the phosphodiester linkages, resulting in spontaneous hydrolysis of nucleosides from the DNA molecule (Brookes and Lawley, 1964; Lawley, 1966). This depurination causes further instability of the sugar-phosphate chain and leads to chain scission, which, in the absence of a repair mechanism, might cause the deletion of genetic material.

The activity of many nitrosamines in the alkylation of nucleic acids is well documented (see Magee and Schoental, 1964; Magee and Barnes, 1967). In the context of biological activity and the alkylation of nucleic acids it is interesting to note the difference between the activities of n-butylmethylnitrosamine (nBMN) and tert-butylmethylnitrosamine (tertBMN). Methylation of rat liver RNA occurred with nBMN only (Magee and Lee, 1964), as might be expected from consideration of the inability of tertBMN to undergo alpha-oxidation, to yield diazomethane or diazobutane as alkylating intermediates (Magee and Barnes, 1967). Significantly only nBMN was carcinogenic, causing tumours of the liver, lung and oesophagus (Druckrey *et al.*, 1967). The precise relationship between nucleic acid alkylation and carcinogenesis is not understood, however.

Reaction of MNNG with adenosine and guanosine *in vitro* was reported by Rau and Lingens (1967) with a yield of 1- and 3-methyladenosine and 7-methylguanosine, respectively.

4. Nucleic acid biosynthesis

A consideration that agents might interfere with the biosynthesis of nucleic acids is of equal significance to any direct action which they may show with nucleic acids. The biosynthesis of nucleic acids has been reviewed by Davidson (1965) and Smellie (1965) and may be conveniently considered to take place in two stages, first, the formation of ribo- or deoxyribonucleosides and their phosphorylation, and second, the polymerisation of units into polynucleotides. The following summary incorporates the salient features presented in these reviews and refers particularly to the synthesis of DNA. The processes of biosynthesis are shown diagrammatically in Fig. 13.

a. Purine biosynthesis

Information on the biosynthesis of purines was obtained through studies with radioactive compounds. Analysis was made of the radioactively labelled degradation products of purines extracted from the nucleic acids of tissue from, and of the uric acid excreted by, pigeons fed radioactive compounds. The process is now understood to involve a complex of enzyme systems and a variety of substrates. Basically the system involves glycine, glutamine, CO_2, aspartate, fumarate and ribose-5'-monophosphate, with ATP as the energy source and Mg^{2+} and folic acid derivatives as co-factors. The product of this reaction is inosine-5'-monophosphate (IMP), which, after suitable modification, gives rise to adenosine-5'- and guanosine-5'-monophosphate (AMP and GMP, respectively, see Fig.14).

b. Pyrimidine biosynthesis

The pathway of pyrimidine biosynthesis was determined mainly from work with microorganisms, but the same system appears to operate

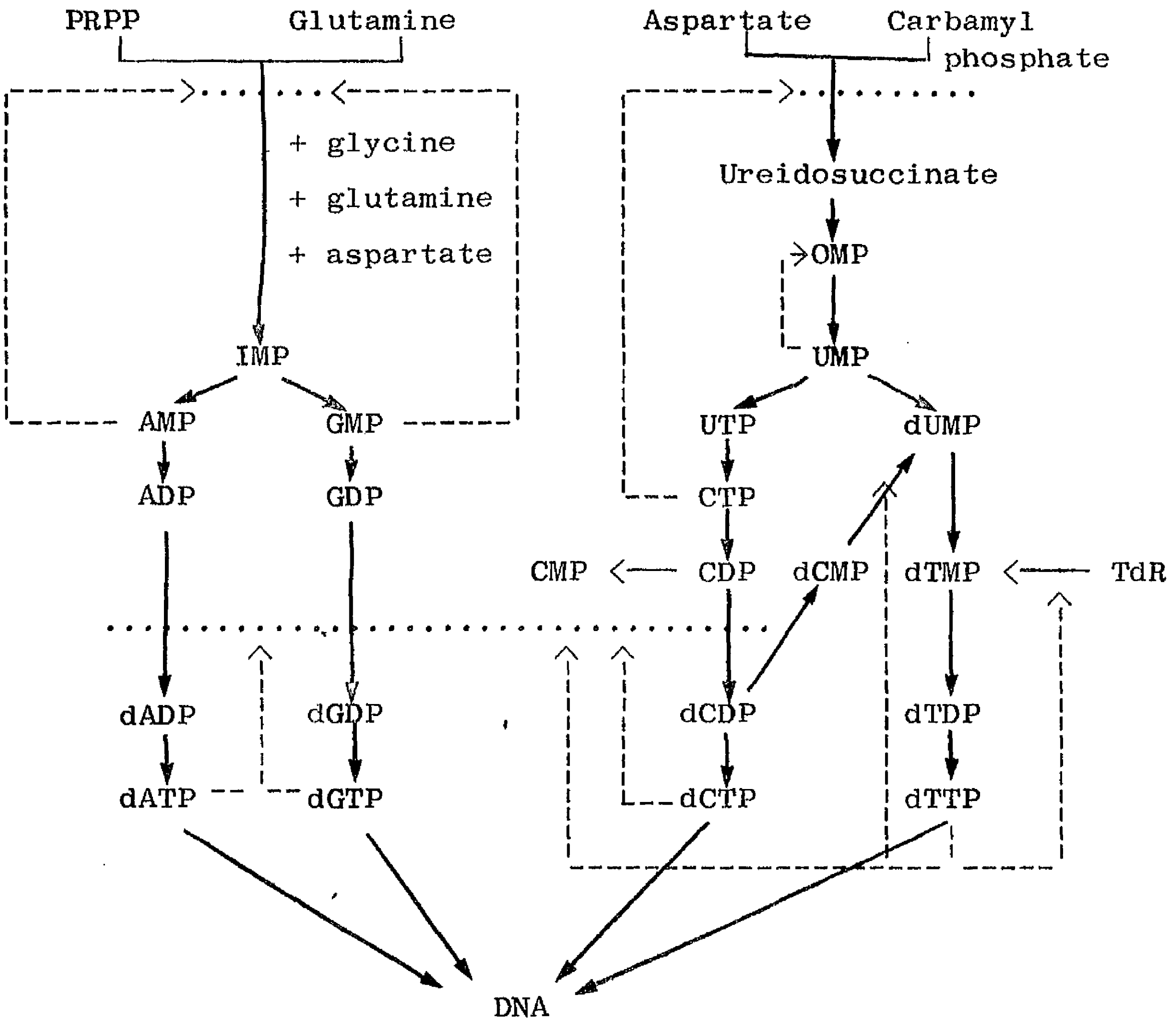


Figure 13. Pathways involved in the biosynthesis of the deoxyribonucleoside triphosphates.

Feed-back mechanisms are indicated by dotted lines.

(Davidson, 1965.)

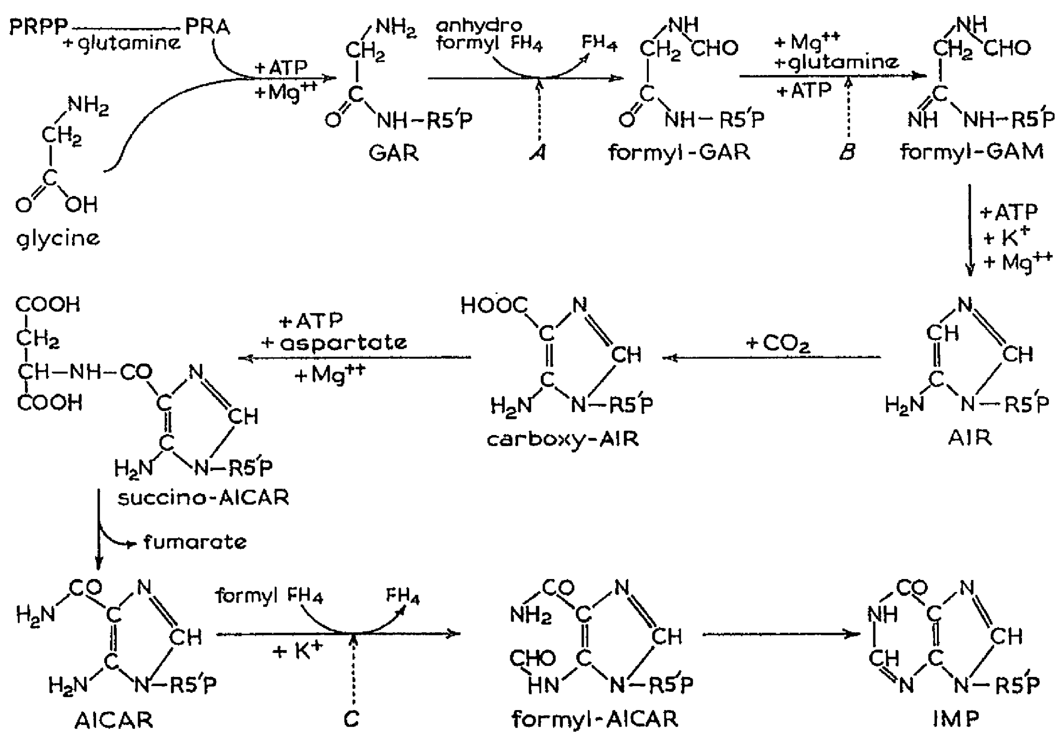


Figure 14. Biosynthetic pathway of purines.

(Davidson, 1965.)

in mammalian cells (Davidson, 1965). Aspartate and carbamoyl phosphate combine to form orotic acid which is converted to uridine-5'-monophosphate (UMP) by the addition of riboso-5'-monophosphate (Fig.15). Conversion of the uridylic residue to the cytidine derivative, by amination at C-4, occurs at the triphosphate level (Fig. 13).

Thymidine-5'-monophosphate (dTMP), on the other hand, is derived separately by the introduction of a methyl group at C-5 of dUMP. This reaction is catalysed by a separate enzyme, thymidylate synthetase, with folic acid derivatives as co-factors.

c. Formation of deoyxribonucleotides and their phosphorylation

The deoxyribonucleotides of adenine, guanine and cytosine are formed by the reduction of the respective ribonucleotides, at the diphosphate level, for subsequent formation of deoxyribonucleoside triphosphates to be incorporated into DNA. The phosphorylation of both ribo- and deoxyribonucleosides is catalysed by enzymes known collectively as "kinases", although a separate kinase is involved in each step. The formation of dUMP, however, is exceptional in that it is formed by deamination of dCMP, derived from dCDP. The deamination is catalysed by the enzyme deoxycytidylate deaminase.

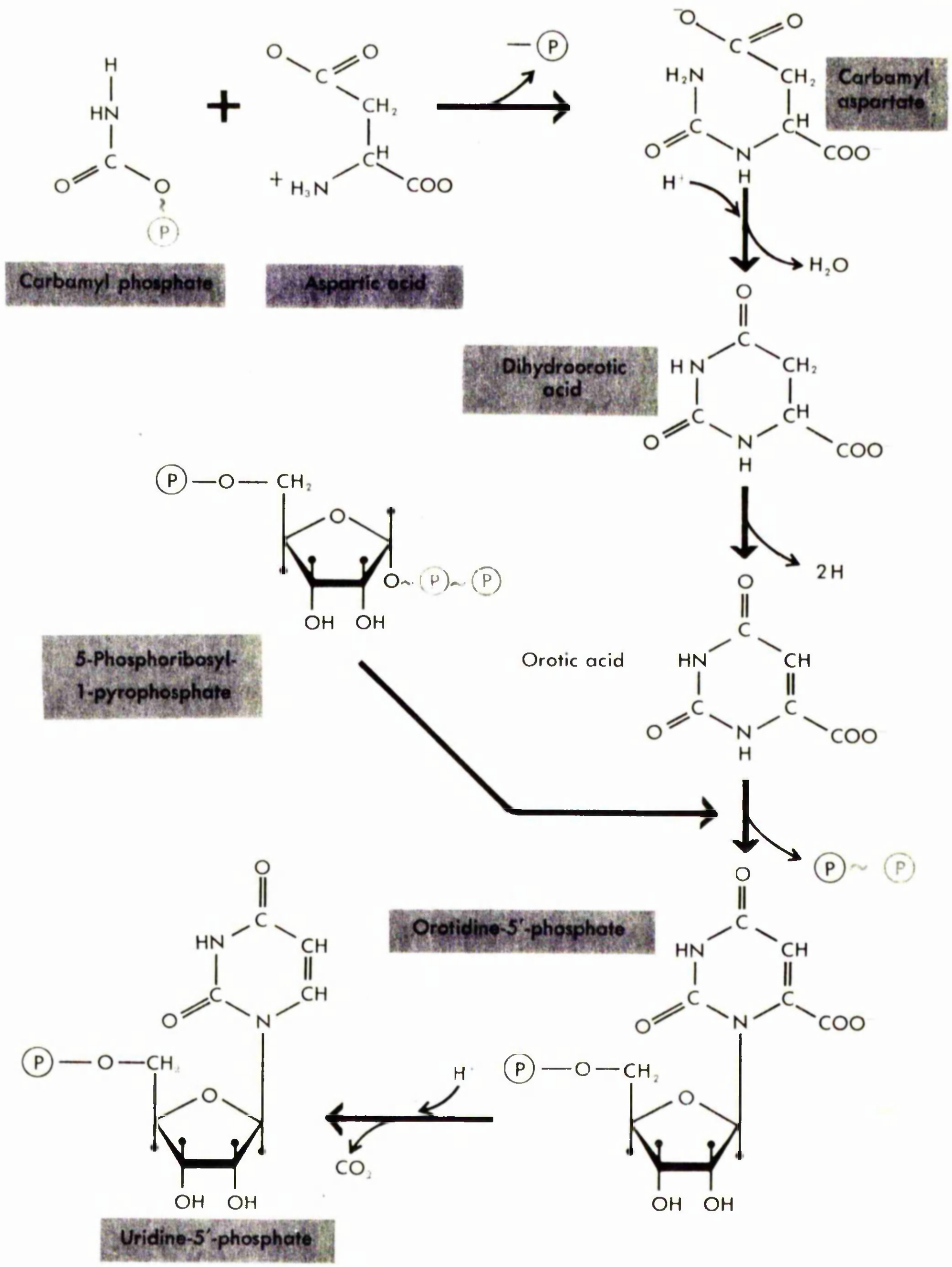
The formation of dTMP from dUMP is described above, (b), and its further phosphorylation is catalysed by thymidylate kinases. It is important to mention here that a separate kinase exists for the conversion of thymidine (TdR) to dTMP. This thymidine kinase, and the thymidylate kinases, have a significant activity in the preparation of exogenous thymidine for incorporation into DNA of cells, as in experiments of exposure to radioactive thymidine.

d. The control of nucleotide biosynthesis

Although the controlling influences which determine the entry of cells into the period of DNA synthesis are not known there is information relating to the regulation of the synthesis of DNA precursors. This has accumulated from work, for example, with cell extracts <u>in vitro</u>, or

Figure 15. Biosynthetic pathway of uridine-5-phosphate.

(from Watson, 1965).



Carbamyl phosphate
Aspartic acid
Carbamyl aspartate
Dihydroorotic acid
5-Phosphoribosyl-1-pyrophosphate
Orotic acid
Orotidine-5'-phosphate
Uridine-5'-phosphate

with cultured mammalian cells, and consists of a number of "feed-back" mechanisms of a positive or negative type. An example of the former is given by the induction, with thymidine, of thymidine kinase for its conversion to dTMP, to be subsequently incorporated into DNA by the usual procedure. Negative feed-back, however, involves a reduction in the amount, or activity, of enzymes by the end-products of the reaction. It is observed, for example, in the inhibition by deoxyribonucleoside triphosphates of the conversion, by ribonucleotide reductases, of ribonucleoside diphosphates to the deoxy- derivatives. The major influence in this latter inhibition appears to be dTTP, an accumulation of which in cells depresses the formation of both purine and pyrimidine deoxyribonucleotides.

These inhibitory effects are represented on the diagram of DNA synthesis (Fig. 13) by dotted lines. The possibility that other enzyme pathways, for example, of deoxycytidylate deaminase in the formation of dUMP, may also contribute to the regulation of DNA precursor synthesis cannot be excluded.

e. Polymerisation of units into deoxyribonucleic acid

Kornberg, Lehman, Bessman and Simms (1956) described an enzyme, extracted from <u>Escherichia coli</u>, which caused the polymerisation of deoxyribonucleotides under appropriate conditions. An enzyme with similar activity was reported in extracts of Ehrlich ascites tumour cells by Davidson, Smellie, Keir and McArdle (1958). Such enzymes are known as DNA polymerases (Deoxyribonucleotide; DNA nucleotidyltransferase, E.C.2.7.7.7.) and critical reviews have been given of the enzyme activity extracted from mammalian cells (Keir, 1965) and from tumour cells (Furlong, 1967). Purification of the bacterial enzyme has been achieved by Kornberg and his colleagues and of the enzyme from mammalian cells by others. For example, Bollum (1960) utilises acid precipitation, ammonium sulphate fractionation and passage through DEAE-cellulose to separate the DNA polymerase of calf thymus from degradative enzymes.

The assay of activity involves, for example, the demonstration of incorporation into DNA of individual, radioactively labelled, thymidylate units from thymidine-5'-triphosphate. This reaction occurs in the presence of enzyme, DNA "primer", the other three deoxyribonucleoside triphosphates (dATP, dCTP, dGTP), divalent cation (usually Mg^{2+}) when the mixture at controlled pH (7.5) is incubated at 37^{o}. The product of the reaction has been characterised to be a replica of the template "primer" DNA in two ways, namely, the similarities between the molar proportions of the nucleic acid bases in the primer and the product, and also from the analysis of nearest neighbour frequency of the bases after degradation of the product (see Smellie, 1965). This latter procedure determines the frequency with which any given nucleotide occurs adjacent to any other and may provide an indication of similarity between nucleotide sequences of a DNA molecule.

The physical structure of the product of DNA polymerase reaction was studied by Schildkraut, Richardson and Kornberg (1964). A branched structure, "pleated" or as "multiple hairpins" was visualised on electron micrographs and the molecule possessed an unusual capacity to re-assume a helical structure after denaturation treatment. More recently, however, the synthesis in vitro of biologically active molecules of bacteriophage DNA has been reported (Goulian, Kornberg and Sinsheimer, 1967).

The precise mode of action of DNA polymerase, and also its molecular configuration, are not known. Moreover, the exact relationship of the extracted DNA polymerase to the enzyme functioning within the cell is uncertain. This uncertainty follows from considerations of the DNA primer molecule, of the requirement for one, or for four deoxyribonucleoside triphosphates, in the reaction, and also of the ability to extract DNA polymerase activity from both the cytoplasm and the nucleus of the cells.

The purified bacterial DNA polymerase utilises denatured and native DNA primer equally well (Richardson, Schildkraut, Aposhian and Kornberg, 1964). The enzyme preparations from most mammalian sources appear to operate more efficiently with denatured rather than native

DNA primer. This relationship does not hold for some systems, such as those extracted from regenerating rat liver (Mantsavinos, 1964). No satisfactory explanation has been given for what amounts, in many cases, to a preference by DNA polymerase for a substrate not identical with the in vivo molecule.

A distinction has been emphasised by Keir (1965) between DNA polymerase reaction that is "replicative" and that which is "end-addition" only. In the former reaction the DNA primer acts as "template", promoting incorporation by complementary base-pairing according to Watson-Crick principles (see I, A.). In the "end-addition" reaction deoxyribonucleotides are polymerised, but not in accordance with any base sequence. Another distinguishing feature between these reactions is that for "replicative" activity the presence of all four deoxyribonucleotides (dATP, dCTP, dGTP and dTTP) is obligatory, whilst the "end addition" reaction occurs (though to a lesser extent) in the presence of only one.

It is not understood why DNA polymerase activity is present within both cytoplasmic and nuclear extracts of cells. The possibility that the activity in the cytoplasmic extract represents precursor units of the enzyme is supported by the results of experiments in which the yield of activity in cytoplasmic extracts of cells decreases, and that of the nuclear extract increases, in cell populations stimulated to undergo DNA synthesis (see Keir, 1965).

Reference has already been made (I, B.2.) to a similar enzyme, DNA-dependent RNA polymerase, which utilises DNA as the primer (template) for the synthesis of RNA.

5. The biological experimental system

The cultivation of mammalian cells in vitro is a commonly accepted practice of current research in the biological sciences. A comprehensive account of the development of various procedures in the exercise of cell culture in vitro is recorded in text books on the subject, such as those by Willmer (1965) or Paul (1964). From the variety of techniques which can be used, the cultivation of cells dispersed as a monolayer on glass or polystyrene substrates and deriving their nutrition from an overlayer of fluid medium, was chosen for the present study.

A collection of different cell cultures is maintained in the Repository of the American Type Culture Collection, in Maryland, USA. One type of culture is the cell "line", which shows an apparent immortality in perpetual cell division. Stocks of such cells are maintained there under standardised conditions of nutrition, and freedom from infection by viruses, for example. The HeLa line, derived from human cervical tissue, and the Chang line, derived from human liver, are two types of cell lines so maintained. Among the advantages attached to the use of such cells are the apparent reproducibility, on separate occasions, of cell populations for comparative studies, and also the range of size of cultures, from single cells to bulk cultivation, which can be studied. The primary disadvantage to their employment is the uncertainty that attends the application to the intact animal of results obtained with the cultured cells. The artificial conditions for the maintenance of viability and the removal of cells from the influence of contiguous and differentiated cells may themselves have so altered the homeostasis of the cell as to make comparison with the in vivo cell incorrect.

Nevertheless, considerable contributions towards the appreciation of biological activity by various agents have been made from work with such in vitro cell systems. For example, observations have been made on the cell "colonies" arising from the multiplication of single cells plated according to techniques outlined by Puck and Marcus (1955). Stoker (1963) commented upon the irregularities of cell growth patterns in colonies derived from hamster cells that had been infected with the polyoma virus, which is carcinogenic (see Rowson, 1966). The irregularities were correlated with the ability of such cells to induce tumours when they were reinoculated into animals. This morphological change in the colonies of cells is referred to as "neoplastic transformation". Similar transformation in cells exposed to other agents has been reported, for example, carcinogenic hydrocarbons (Berwald and Sachs, 1963; Heidelberger and Type, 1967) and the carcinogenic nitrosamine, N-methyl-N-nitrosourea (Sanders and Burford, 1967). The precise mechanism underlying this change is not understood.

Another aspect of study in cultured mammalian cells follows from the observation in plant cells (Howard and Pelc, 1953) and cultures of human bone marrow cells (Lathja, Oliver, Berry and Noyes, 1958) that the period between successive cell divisions could be divided into phases, the whole constituting a "life cycle". Following mitosis, (M), there is an interval, (G_1), before the cells begin to synthesise DNA, (S). The S phase is recognised on autoradiographs by the incorporation of radioactively labelled precursor (usually thymidine) into DNA of the nucleus. A second interval, (G_2), then follows before the cell enters into a second mitosis, (M). Work has been carried out to characterise the metabolic events of the different phase, G_1, S, G_2 and M of the life cycle, such as the protein synthesis required for a cell to enter into DNA synthesis (Terasima and Yasukawa, 1966; Donnelly and Siskin, 1967).

Techniques have been developed to localise the action of a given metabolic inhibitor to a specific period in one of the phases of the cell life cycle. The method of Puck and Steffen (1963), for example, makes use of Colcemide, a chemical which arrests the passage of cells through mitosis at metaphase, when the chromosomes are clearly delineated. The accumulation of the arrested cells in an exponentially growing population of cells can be assessed on suitable preparations of samples taken at intervals after the addition of Colcemide. From the rate of accumulation, and the timing of appearance of labelled metaphase cells in populations treated also with radioactive thymidine, suitable calculations provide estimates of the duration of the different phases of the life cycle. Alterations to the rate of accumulation caused by treatment with an agent may be used to calculate the period of the life cycle of the cell at which there exists an event sensitive to the agent. The

The agent of the experiments of Puck and Steffen (1963) was X-irradiation.

The incorporation (per unit time) of radioactive precursor into DNA, RNA or protein may be estimated in suitable extractions of the cells to provide a quantitative assessment of the rate of metabolic processes and their inhibition in cultured cells.

B. Methods and Materials

1. General preparative procedures

a. Preparation of Glassware

Roux bottles, medicine bottles, Universal containers and pipettes were treated according to procedures used routinely in the Department of Biochemistry, Glasgow University.

An initial tap-water rinse was followed by an overnight soak in Decon 75 (Medical Pharmaceutical Developments, Sussex, England) at 1:150 dilution in water; a second tap-water rinse was followed by a rinse with de-ionised (Elgestat: Elga Products Ltd., London) water before the glassware was over-dried. Pipettes were rinsed in an automatic, syphonic rinser.

Bottle tops were treated separately. Liners were removed and boiled in Decon solution (1:150) for ten minutes; metal parts were boiled in $NaHCO_3$(0.2M); both were separately rinsed in tap-water before they were boiled in de-ionised (Elgestat) water and then dried.

Winchester bottles ($2\frac{1}{2}$ 1) were washed according to the methods of Martin, Malec, Sved and Work (1961). Calgon (20 g. Albright and Wilson Ltd., England) was mixed with sodium metasilicate (180 g.) in water (2,270 ml.) and the mixture filtered for 24 hours. The bottles were soaked overnight in diluted (1:100 in water) silicate solution, rinsed with tap-water and then soaked in 0.1 N Hydrochloric acid for thirty minutes before they were rinsed with de-ionised (Elgestat) water and dried in the oven.

Pipettes were sterilised at 160^{o} for two hours; all other glassware was steam sterilised at 15 lbs. for thirty minutes.

b. Growth medium

Medium N 16 (Puck, Cieciura and Robinson, 1958) and medium NCTC 109 (McQuilkin, Evans and Earle, 1957) were purchased from Microbiological Associates (Bethesda, Maryland, USA). Minimal essential medium (Eagle, 1955) was supplied by Burroughs Wellcome & Co., London, England.

Foetal calf serum was obtained from Flow Laboratories, Irvine, Scotland.

The constituents of media N 16, NCTC 109 and minimal essential medium (Eagle) are given in Tables 10, 11 and 12, respectively.

c. Solutions and supplements to media

All chemicals used in the preparation of solutions were Analar grade, or equivalent, obtained from British Drug Houses, Poole, England.

(i) Balanced salt solution. The inorganic salts solution (BSS) according to Earle, as described in Flow Laboratory Catalogue (1967), comprised per litre:-

NaCL	6800 mg.	$NaH_2PO_4.2H_2O$	140 mg.
KCl	400 mg.	$MgSO_4.7H_2O$	200 mg.
glucose	1000 mg.	$CaCl_2.2H_2O$	264 mg.
$NaHCO_3$	2000 mg.	phenol red	15 mg.

The solution was prepared without $NaHCO_3$ and sterilised by autoclave (15 lbs., 30 min.) for storage at room temperature. $NaHCO_3$ solution (6.6% w/v), prepared and sterilised separately, was added as required.

(ii) Saline D2. This salt solution was prepared exactly according to Ham and Puck (1962). It does not contain $NaHCO_3$ and therefore does not become excessively alkaline on exposure to air. Cells suspended in this solution for plating procedures were therefore protected from critical changes of pH. The final concentration of salts was, per litre solution:-

NaCl	8000 mg.	$Na_2HPO_4.7H_2O$	45 mg.
KCl	400 mg.	KH_2PO_4	30 mg.
Glucose	1000 mg.	$CaCl_2.2H_2O$	16 mg.
	phenol red	1.2 mg.	

(iii) Tryptose phosphate broth. The following compounds were dissolved in one litre of glass-distilled water:-

Table 10. Composition of Medium N16 according to Puck, Cieciura and Robinson, 1958.

Synthetic nutrient solution			
Constituent	g/l.	Constituent	g/l.
L-Arginine HCl	0.0375	Glutamine	0.20
L-Histidine HCl	0.0375	L-Tyrosine	0.040
L-Lysine HCl	0.080	L-Cystine	0.0075
L-Tryptophane	0.020	Hypoxanthine	0.025
β-Phenyl-L-alanine	0.025	Thiamine HCl	0.0050
L-Methionine	0.025	Riboflavin	0.00050
L-Threonine	0.0375	Pyridoxin HCl	0.00050
L-Leucine	0.025	Folic acid	0.00010
DL-Isoleucine	0.025	Biotin	0.00010
DL-Valine	0.050	Choline	0.0030
L-Glutamic	0.075	Ca pantothenate	0.0030
L-Aspartic	0.0300	Nicotinamide	0.0030
L-Proline	0.025	i-Inositol	0.0010
Glycine	0.100		
Salt solution			
NaCl	7.40	KH_2PO_4	0.083
KCl	0.285	$NaHCO_3$	1.20
$MgSO_4 \cdot 7H_2O$	0.154	Glucose	1.10
$CaCl_2 \cdot 2H_2O$	0.016	Phenol Red	0.0012
$Na_2HPO_4 \cdot 7H_2O$	0.29		

Table 11. Composition of Medium NCTC 109 according to Evans, Bryant, Kerr and Schilling, 1964.

Amino acids			
Constituent	Conc. (mg/l)	Constituent	Conc. (mg/l)
L-alanine	31.48	L-leucine	20.44
L-a-amino-n-butyric acid	5.51	L-lysine·HCl	38.43
L-arginine·HCl	31.16	L-methionine	4.44
L-asparagine·H_2O	9.19	L-ornithine·HCl	9.41
L-aspartic acid	9.91	L-phenylalanine	16.53
L-cystine	10.49	L-proline	6.13
D-glucosamine·HCl	3.85	L-serine	10.75
L-glutamic acid	8.26	L-taurine	4.18
L-glutamine	135.73	L-threonine	18.93
glycine	13.51	L-tryptophan	17.50
L-histidine·HCl·H_2O	26.65	L-tyrosine	16.44
hydroxy-L-proline	4.09	L-valine	25.00
L-isoleucine	18.04		
Vitamins			
Thiamine·HCl	0.025	choline chloride	1.25
riboflavin	0.025	vitamin B_{12}	10.0
pyridoxine·HCl	0.0625	i-inositol	0.125
pyridoxal·HCl	0.0625	p-aminobenzoic acid	0.125
niacin	0.0625	vitamin A (cryst.alcohol)	0.25
niacinamide	0.0625	calciferol (vitamin D_2)	0.25
d-pantothenate, calcium	0.025	menadione (vitamin K)	0.025
d-biotin	0.025	a-tocopherol phosphate,	
folic acid	0.025	disodium salt (Vit. E)	0.025
Coenzymes			
DPN (diphosphopyridine		TPP (cocarboxylase)	1.0
nucleotide	7.0	FAD (flavin adenine	
TPN (triphosphopyridine		dinucleotide)	1.0
nucleotide, monosodium	1.0	UTP (uridine triphosphate,	
CoA (coenzyme A)	2.5	sodium	1.0
Reducing agents			
glutathione, monosodium	10	L-cysteine·HCl	260
ascorbic acid	50		
Nucleic acid derivatives			
deoxyadenosine	10	thymidine	10
deoxycytidine·HCl	10	5-methylcytosine	0.1
deoxyguanosine	10		

Constituent	Conc. (mg/l.)	Constituent	Conc. (mg/l.)
Lipid sources			
methyl linoleate	1.0	cholesterol	2.0
methyl linolenate	1.0	Tween 80	12.5
methyl arachidonate	1.0		
Glucose derivatives and sodium acetate			
D-glucuronolactone	1.8	sodium acetate$\cdot 3H_2O$	50
sodium glucuronate$\cdot H_2O$	1.8		
Salt solution			
NaCl	6800	$NaHCO_3$	2200
KCl	400	D-glucose	1000
$CaCl_2$	200	phenol red sodium salt	20
$MgSO_4$	100	ethyl alcohol	40
$NaH_2PO_4 \cdot H_2O$	140		

Table 11 (Continued)

Table 12. Minimal essential medium (Eagle) as supplied by Burroughs Wellcome, Ltd.

The medium is diluted ten times in glass distilled water.

Constituents	mg./l.
<u>Amino Acids</u>	
l-argine hydrochloride	1250.0
l-cystine	240.0
l-histidine hydrochloride	400.0
dl-isoleucine	1040.0
dl-leucine	1040.0
l-lysine hydrochloride	700.0
dl-methionine	300.0
dl-phenylalanine	640.0
dl-threonine	960.0
dl-tryptophane	200.0
dl-valine	920.0
l-tyrosine	360.0
<u>Vitamins</u>	
Choline chloride	10.0
Folic acid	10.0
Inositol	20.0
Nicotinamide	10.0
Calcium pantothenate	10.0
Pyridoxal hydrochloride	10.0
Riboflavin	10.0
Aneurine hydrochloride	10.0
<u>Inorganic Salts</u>	
Sodium chloride ($NaCl$)	68,000.0
Potassium chloride (KCl)	4000.0
Calcium chloride ($CaCl_2$)	2000.0
Magnesium sulphate ($MgSO4.7H_2O$)	2000.0
Sodium phosphate ($NaH_2PO_4.2H_2O$)	1500.0
<u>Miscellaneous</u>	
Glucose	10,000.0
l-glutamine	2920.0
Phenol red	100.0

Bacto-tryptose (Difco Labs., Detroit, Michigan, USA) .... 20 g.

Glucose .... 2 g.

Sodium chloride .... 5 g.

Disodium hydrogen phosphate .... 2.5 g.

The solution was dispensed as 20 ml. aliquots into Universal containers for steam sterilisation at 15 lbs. for 30 min.

(iv) Trypsin solution. The following were dissolved in glass-distilled water:-

Trypsin (Difco Labs.; 1:250) .... 2.5 g.

Sodium citrate .... 2.96 g.

Sodium chloride .... 6.00 g.

The volume was made up to 1 litre and the pH adjusted to 7.8. The solution was sterilised by filtration (Millipore (U.K.) Ltd., Wembley, Middlesex, England; 142 mm., pore size 0.22 μ) and dispensed as 100 ml. aliquots in bottles for storage at -20°. All batches were sterility checked before use.

(v) Cell counting fluid. The following were dissolved in glass-distilled water:-

Sodium chloride .... 7 g.

Citric acid .... 10 g.

Mercuric chloride .... 1 g.

The volume was made up to 1 litre and the solution filtered through sintered glass.

(vi) Haematoxylin stain. Harris's alum haematoxylin stain was prepared and used according to the recommendations of Culling (1957). The stain was filtered before use on each occasion.

(vii) Phosphate buffer solutions. These were prepared according to Gomori (1955) at pH 7.4., pH 7.0 and pH 6.0, at 0.1 M strength for sterilisation by autoclave and storage at 4°. Buffers at 0.01 M were prepared by dilution.

(viii) Preparation of MNNG solution. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was purchased from Aldrich Chemical Co., Inc., (Milwaukee, Wisconsin, USA) and was dissolved at 0.02 M in 0.01 M phosphate buffer, pH 6, by shaking at room temperature in the dark. The solution was sterilised by filtration (Nalge Co., Inc., Rochester, USA., Filter unit, plain membrane, pore size 0.22 μ) and dispensed as 1 ml. aliquots in Bijou bottles, wrapped in tin-foil, for storage at -20°. Once thawed, the sample was never used again. Appropriate dilutions in 0.01 M phosphate buffer were made such that additions of 0.1 ml. gave the required concentration.

(ix) Colcemid solution. Colcemid (N-deacetyl-N-methylcolchicine, Ciba Laboratories, Surrey, England) was dissolved in BSS at 500 μg./ml. for filter sterilisation (Nalge Filter Unit, 0.22 μ pore size) and storage at -20°. Appropriate dilutions were made in (sterile) BSS for addition to growth medium.

2. <u>Measurement of MNNG half-life</u>.

The approximate half-life of MNNG in the experiments of the present study was calculated from the rate of disappearance of the absorption band near 400 mμ associated with the nitroso group (Lawley, 1968) in a solution of MNNG in growth medium ($PNFC_{20}$, see III, B.3.a) maintained at 37° in the dark. Measurements were made on a Unicam SP 800 Spectrometer (Unicam Instruments Ltd., Cambridge, England) fitted with an attachment (SP 825) for automatic re-scanning at fixed intervals. Cuvette temperature was maintained at 37° with a thermostatically controlled aluminium block.

Aliquots of medium, either fresh, or conditioned by incubation with growing cells, were removed from the incubator and allowed to equilibrate in stoppered, quartz cuvettes (Unicam 1 cm. light path) mounted in the aluminium block, for 10 min. An aliquot of a freshly prepared 50 mM solution of MNNG in ethanol was used to make the solution in the test cuvette 5 mM with respect to MNNG. Ethanol alone was added to the control cuvette. The absorption spectrum was recorded every 15 min. thereafter.

Measurements of initial and final pH of the reaction mixture, test and control, were made with an Astrup pH Micrometer (Astrup Micro Equipment, Copenhagen, Denmark.).

Results. The absorption spectrum obtained in a typical experiment with fresh medium is shown in Fig. 16 from which data a half-life of the order of 90 min. was calculated. The initial pH was 7.45 and the final pH was 7.08 (control, final pH 7.4). Another experiment, with medium incubated with cells for 18 hr., gave a value of 85 min., but with medium incubated with cells for 60 hours the half-life was increased to 150 min. The pH of the latter "old" medium was initially 7.2 and fell to 6.88. The greater stability of MNNG in acid (Lawley, 1968) is one factor which would contribute to this observed increase of half-life. However, since cells in this study were routinely exposed to MNNG after 18-20 hr. incubation, the value of 90 min. half-life was accepted as applicable to the present study. This value is in agreement with data of MNNG half-life at neutral pH reported by Lawley (1968) and by McCalla and Reuvers (1968).

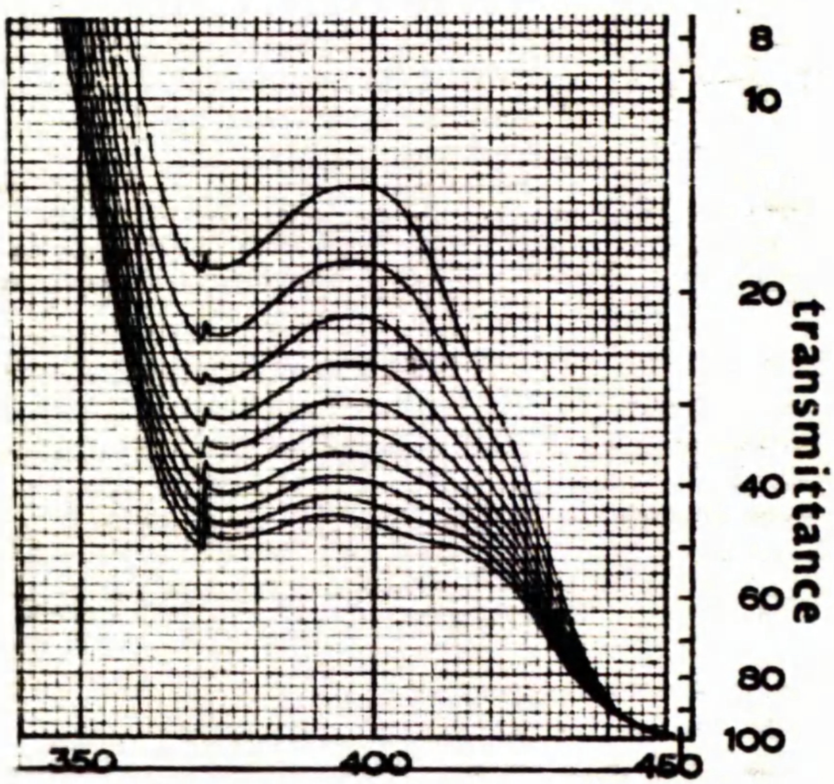
3. Cell cultivation procedures

The cells used in this study were obtained from the American Type Culture Collection Cell Repository (12301, Parklawn Drive, Rockville, Maryland, USA). The fibroblast-like cell line, Don (CCL 16), derived from an adult, male Chinese hamster (*Cricetulus griseus*) was cultured as follows.

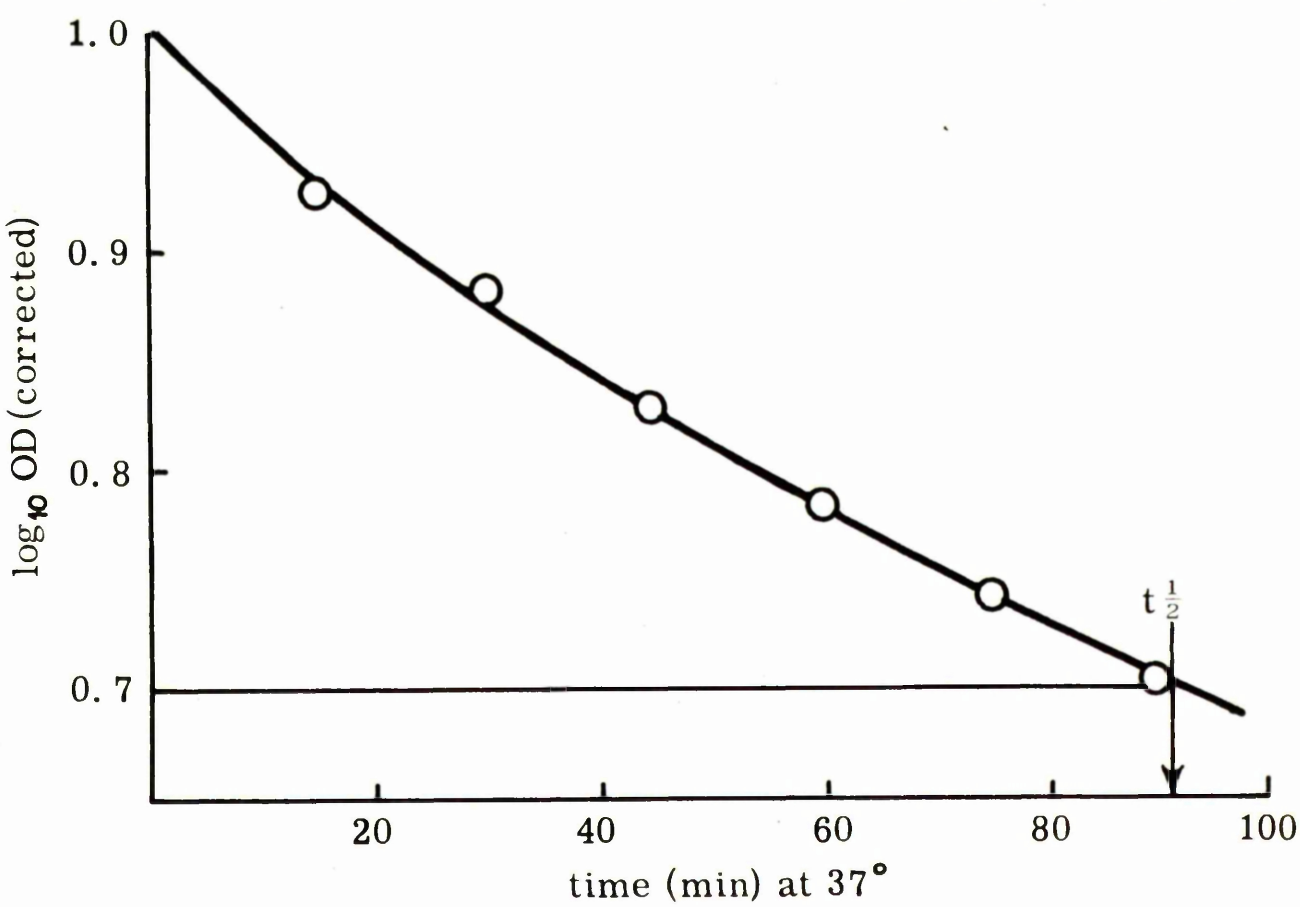
a. Maintenance, subculture and cell counting techniques. The cells were maintained in monolayer culture on the flat surface of Roux bottles

Figure 16. Stability of N-methyl-N'-nitro-N-nitrosoguanidine in growth medium.

The disappearance of the absorption maximum at 399 mμ with time is shown for a solution of MNNG prepared and examined as described in the text. The optical density values (corrected), for the same run, are plotted on a logarithmic scale against time of incubation to show the derivation of half-life value (approximate).



8
10
20
40
60
80
100
transmittance
350
400
450



1.0
0.9
0.8
0.7
$\log_{10}$ OD (corrected)
$t\frac{1}{2}$
20
40
60
80
100
time (min) at 37°

charged with approximately 50 ml. medium and gassed with a mixture of 5% CO_2 in air; the bottles were incubated at 37^o. The medium was composed of Puck's N 16 (60% v/v), NCTC 109 (20% v/v) and foetal calf serum (20% v/v) and is referred to hereafter as $PNFC_{20}$. The medium was changed according to acidity and the cells subcultured at confluence. Cells were not maintained beyond approximately twenty subcultures; fresh cultures were established from stocks of cells ($2x10^6$ cells in 1 ml. $PNFC_{20}$ supplemented with 5% glycerol) stored in ampoules in liquid nitrogen (-196^o).

To subculture cells, the medium was removed by pipette and 5 ml. trypsin solution (III, B.1.c.iv) was introduced. After brief exposure of the cells, by swirling the solution over them, the trypsin solution was pipetted off and the Roux flask returned to incubate at 37^o. The cells became detached within a few minutes and were washed off the glass surface into fresh medium (10-20 ml.), which was pipetted up and down, gently, to encourage dispersion into single cells. The suspension was transferred to a Universal container and aliquots from appropriate dilutions in medium were diluted 1:50 (v/v) in counting fluid (III, B.1.c.v.) for electronic assessment of cell number (Model D, Coulter Electronics Ltd., St. Albans, England).

Duplicate samples were prepared and the cell dilution mixed by pipette until sequential counts differed by less than 5%. After calibration for "coincidence" counts against the manufacturer's tables, the mean cell count per ml. was calculated, and a suitable volume, usually to contain $5x10^6$ cells, was introduced into a fresh Roux bottle charged with 50 ml. $PNFC_{20}$. The bottle was then gassed with 5% CO_2 in air before it was incubated at 37^o.

b. Examination of cells for contamination by Mycoplasma species.

Plastic dishes (Esco Rubber Ltd., London, grade AA, 60 mm. pre-sterilised) containing cover-slips (Chance, England, 22x22 mm., No.1) were inoculated with $0.5x10^6$ cells contained in 5 ml. $PNFC_{20}$ and incubated for at least 24 hr. at 37^o in a humidified atmosphere of 5% CO_2

in air. The cells were then treated according to Fogh and Fogh (1964) for the demonstration of intracellular Mycoplasma organisms.

The medium was replaced with 4 ml. sodium citrate solution (0.6% w/v) and 1 ml. water added dropwise. The preparation was left for 10 min. before fixation was begun by slow addition of ethanol/ acetic acid (3:1)(5 ml.). This solution was replaced with undiluted fixative and the preparation left undisturbed for at least 30 min. The coverslips (duplicates) were subsequently air-dried, to be stained with orcein (2% w/v in 60% glacial acetic acid) for 5 min., washed three times with 5 ml. ethanol and mounted. Examination of the cells was made under the oil-immersion objective (x 100, phase contrast).

Contaminated cultures were discarded.

c. Cells cultured in plastic Petri dishes

The following procedure was used to obtain multiple numbers of comparable cell populations for experimental study. Cells were harvested from growth in Roux bottles by procedures of trypsinisation already outlined (a., above). The cell concentration was determined (Coulter, Model D, a., above) and an appropriate volume diluted into a suitable bulk quantity of growth medium ($PNFC_{20}$). After thorough mixing, 5 ml. aliquots were dispensed by pipette into plastic Petri dishes (Esco, 60 mm.) which were immediately introduced into a humidified atmosphere of 5% CO_2 in air in an incubator at 37°.

Alternatively, the cells were harvested in Saline D2 (III, B.1. c.ii), supplemented with 10% (v/v) foetal calf serum, to be centrifuged (600 g. for 5 min.) and then resuspended in Saline D2 for determination of cell number, as above. The volume of suspension was then adjusted so that aliquots of 100 or 200 µl. (Marburg Micropipettes, Germany) delivered the required cell number into dishes (Esco, 60 mm.), already inoculated with 5 ml. $PNFC_{20}$. The dishes were agitated to ensure dispersion of cells before they were incubated as above.

d. Measurement of cell growth

Cell cultures in plastic Petri dishes (Esco, 60 mm.) were set up and incubated, as outlined above (c.). At intervals three dishes were removed for treatment, as follows, to estimate the number of cells per dish. The medium was decanted into a conical, glass centrifuge tube before trypsin solution (1 ml.) was introduced and the dish incubated for a few minutes at 37°. Detachment of cells from the surface was confirmed microscopically before 2 ml. fresh $PNFC_{20}$ was added to arrest tryptic activity. The detached cells were mixed in the medium by Pasteur pipette (Harshaw Chemicals Ltd., Daventry, England) and collected quantitatively, with two 2 ml. plate rinses with BSS, to be combined with the old medium for centrifugation (600 g. for 5 min.) in a graduated centrifuge tube. The cells were resuspended in a suitable volume and an aliquote removed for assessment of cell number (III, B.3.a).

The cell count per plate was calculated by volume adjustment of the cell count per ml.

e. Accumulation of cells at metaphase

Cell cultures in plastic Petri dishes (Esco, 60 mm.) were set up and incubated, as already outlined (c., above). To accumulate cells at metaphase the method outlined by Puck and Steffen (1963) was followed. At intervals after the addition of Colcemid (Ciba Laboratories, 0.2-2 μg./ml. final conc.) to the growth medium the cells were harvested by trypsinisation (d., above). The supernatent fluid after centrifugation at 4° (400 g. for 5 min.) was withdrawn, the cells were gently resuspended in 1 ml. solution of sodium citrate (0.05%) and sucrose (0.1 M), to promote hypotonic swelling, and introduction of ethanol/acetic acid (3:1) fixative (10 ml.) was immediately started by slow addition down the inside of the tube. The tube was maintained on ice during this period and for a further 30 min. before centrifugation (400 g. for 10 min.) at 4°. The cells were resuspended in 0.1 ml. undiluted fixative (ethanol/acetic acid) and one drop was allowed to air-dry on a coverslip, which was then stained (Harris's haematoxylin) and mounted on a slide for examination under the microscope. Cells in metaphase were readily distinguished, by their delineated chromosomes, under the high-power objective (x40, phase contrast).

f. Assessment of mitotic index

Cultures of cells in plastic dishes (Esco, 60 mm.) containing glass coverslips (Chance, 22x22 mm., No.1) were set up as already described (c., above). After 18 hr. incubation the cell number in three dishes was assessed (d., above) to confirm entry of cells into logarithmic phase of growth, by comparison against standard growth curves (III, C.1.a.). The dishes were made 10 µM with respect to MNNG (MNNG solution, III, B.1.c.viii) and returned to incubate. At intervals after addition of MNNG solution, the dishes were treated as follows. The medium was syphoned off and the cells rinsed twice with 4 ml. BSS. Sodium citrate (0.6% w/v, 4 ml.) was added, followed by dropwise addition of 1 ml. water, and the dish was left for 10 min. before an equal volume of fixative (ethanol/acetic acid, 3:1) was slowly introduced. This mixture was replaced 10 min. later by undiluted fixative (3 ml.) and after 30 min. the coverslips were air-dried, stained with haematoxylin and mounted for examination under the microscope.

Cells in mitosis (anaphase, metaphase and early telophase) were readily distinguished with the high-power objective (x40, phase contrast).

g. Assessment of colony forming ability

The procedure employed in this study was the one outlined by Ham and Puck (1962) and incorporated also the method of irradiated feeder layers (Puck and Marcus, 1955). Cells were harvested after trypsinisation (a., above) from Roux flasks into 10 ml. Saline D2 supplemented with foetal calf serum (10% v/v) and buffered at pH 7.4 with 0.01 M (final) phosphate solution (III, B.1.c.vii). The cell suspension was divided into two aliquots, one of which was irradiated with a lethal dose (^{60}Co, 2000 rad.) at the Department of Radiotherapy, Western Infirmary, Glasgow, Plastic dishes (Esco, 60 mm.) containing 5 ml. $PNFC_{20}$ were inoculated (Marburg Micropipettes, 100 µl.) with $0.5x10^6$ of either irradiated cells, or non-irradiated cells. Both sets of plates were incubated overnight under standard conditions (c., above).

The following day, two control plates of non-irradiated cells were treated for assessment of cell number (d., above) to confirm entry of cells into logarithmic growth, by comparison of counts against standard growth curves. Dishes of non-irradiated cells were then inoculated (Marburg Micropipettes, 100 µl.) with suitable dilutions of MNNG solution (III, B.1.c.viii) to give appropriate final concentrations, and returned to incubate for one hour. The medium was then syphoned off, the plates each rinsed twice with 5 ml. Saline D2 and the cells detached from the surface by trypsinisation (d., above) to be collected in Saline D2 (supplemented as above, c.) for determination of cell number (Coulter, Model D). Suitable dilutions of the cell suspensions in Saline D2 were made in order that aliquots (Marburg Micropipettes, 100 or 200 µl.) dispensed an estimated 500 to 2000 cells into the dishes of irradiated cells already incubated overnight. The dishes were withdrawn briefly from the incubator for inoculation, returned after gentle rocking to ensure even dispersion of cells, and thereafter left undisturbed for at least nine days.

At the end of the incubation period the medium was syphoned off and the plates rinsed twice with 5 ml. Saline D2 before the cells were fixed with ethanol/acetic acid (3:1). The colonies were stained with haematoxylin to assist recognition for counting and examination of colonial morphology by the microscope.

4. <u>Radioactive procedures</u>.

a. Radioactive isotopes

$[^3H]$Thymidine, nominally labelled in the 6-position (specific activity, 17.7 curies/mM), $[^3H]$uridine, labelled specifically in the

5-position, (specific activity, 16.0 curies/mM), [^{3}H]deoxyadenosine, labelled generally (specific activity, 2.4 curies/mM) and [^{14}C]amino acid mixture (L amino acids, labelled uniformly and isolated from Chlorella protein hydrolysate, specific activity, 54 mc/mAtom carbon) were all obtained from the Radiochemical Centre, Amersham, England. [alpha ^{32}P]Thymidine-5'-triphosphate, disodium salt (specific activity, 740 mc/mM) was obtained from Intèrnational Chemical and Nuclear Incorporation, California, USA.

b. Scintillator fluids

Toluene-based scintillator fluid contained, per litre toluene (B.D.H., Analar grade), 5 g. 2,5,Diphenyloxazole (PPO), (Koch Light Laboratories, Bucks., England), 0.3 g. 1,4-bis-(2-(5-phenyloxazolyl))-benzene (POPOP), (Nuclear Enterprises, Edinburgh, Scotland). Hyamine hydroxide, 1 M solution in methanol (Nuclear Enterprises) was added as required to give a final concentration of 5% (v/v).

Dioxane-based scintillator fluid contained, per litre Dioxane (Koch Light, Scintillation grade), 7 g. PPO, 0.3 g. POPOP and 100 g. Napthalene (Nuclear Enterprises, Scintillation grade).

c. Experiments with isotopically labelled precursors

Cell cultures were set up in plastic Petri dishes (Esco, 60 mm.) according to standard procedures (III, B.3.c.) and incubated overnight at 37^o in a humidified atmosphere of 5% CO_2 in air. The following morning the cell population in three dishes was counted (III, B.3.d.) to confirm that normal growth was established, by comparison of counts with standard growth curves. Culture dishes were then inoculated (100 µl.) with appropriate dilutions of MNNG solution in 0.01 M phosphate buffer, pH 6. Controls were inoculated with phosphate buffer alone.

(i) Incorporation of [^{3}H]thymidine into DNA. Control and MNNG treated cell cultures were inoculated with [^{3}H]thymidine (100 µl, solution in BSS; 10 µc/plate) and reincubated for one hour.

The medium was syphoned off and the plate rinsed rapidly, twice with 5 ml. portions of ice-cold BSS before 3 ml. ice-cold 5% trichloracetic acid (TCA) was added. The plates were kept at 4^{o} for at least two hours before the cells were removed from the surface of the plate with a "rubber-policeman", transferred quantitatively (two plate rinses of 3 ml. ice-cold 5% TCA) with a Pasteur pipette and collected on a Millipore filter (Millipore (U.K.) Ltd., London; 25 mm., pore size 0.45 µ) to be subsequently washed with 2x10 ml. portions of ice-cold 5% TCA.

The Millipore filters were dried, immersed in 10 ml. toluene based scintillator fluid (b., above) and counted in a Liquid Scintillation Spectrometer (Packard Instrument Co., Inc., Illinois, USA., Model 4322, or Nuclear Chicago Corporation, Illinois, USA., System 724-725). Counting efficiencies were calculated from quench curves routinely available in the Department of Biochemistry, Glasgow University, obtained from standard $[^{3}H]$ labelled samples.

(ii) Incorporation of $[^{3}H]$uridine into RNA. The procedure outlined above, (i), was used. $[^{3}H]$Uridine (100 µl, solution in BSS; 2.5 µc/plate) replaced $[^{3}H]$ thymidine and the period of exposure was reduced to 20 min.

(iii) Incorporation of $[^{3}H]$ deoxyadenosine into DNA and RNA. Control and MNNG-treated cell cultures were inoculated with $[^{3}H]$ deoxyadenosine (100 µl, $5x10^{-6}$M final concentration; 10 µc/plate) and further incubated for one hour. The medium was syphoned off, the plates rinsed twice with 5 ml. ice-cold BSS before 3 ml. ice-cold 5% TCA was added and the plate stored at 4^{o} overnight. Cells were removed from the surface of the dishes with a "rubber policeman" and the contents of each dish collected quantitatively (2x5 ml. plate rinses, ice-cold 5% TCA) into individual, conical, glass centrifuge tubes. The cells were sedimented by centrifugation at 600 g. for 5 min., washed twice with 10 ml. 5% TCA and finally resuspended in 2 ml. 0.3 N potassium hydroxide. Hydrolysis of RNA was assumed to be complete after one hour at 37^{o} (Munro and Fleck, 1966) in a water bath. Ice-cold 50% TCA was added to make the final concentration 5% with respect to TCA and the centrifuge tubes immersed in ice for one hour.

The acid precipitate was separated by filtration (Millipore, 0.45 μ) and the resulting filtrate collected and stored at 4^{o}. The filters were dried in vials and immersed in 10 ml. toluene based scintillator fluid. Aliquots of the filtrate were added to 10 ml. dioxane based scintillator. Radioactivity was assayed (i, above) to determine incorporation of isotope into DNA (acid precipitate) and RNA (alkaline hydrolysate). A suitable correction was made for the volume of each filtrate to give total RNA radioactivity per sample.

(iv) $[^{14}C]$Amino acid incorporation into protein. The medium in dishes of control and MNNG treated cell cultures was inoculated with $[^{14}C]$amino acids mixture (100 μl, solution in BSS; 0.5 μc/plate) and the dishes incubated for a further 30 min. The cells from each dish were collected on filters (Millipore, 0.45 μ), washed with 2x10 ml. portions of ice-cold 5% TCA and then counted in toluene based scintillator fluid, as before. The efficiency of counting for $[^{14}C]$ was calculated from quench curves routinely available in the Department of Biochemistry, Glasgow University, obtained from $[^{14}C]$ labelled samples.

(v) Incorporation of $[^{3}H]$thymidine into phosphorylated thymidine derivatives. Control and MNNG treated cells were exposed to $[^{3}H]$thymidine (100 μl, solution in BSS; 20 μc/plate) for the second hour of incubation after addition of MNNG solution, then washed twice with 5 ml. ice-cold BSS. The cells were scraped off the surface of the dish with a "rubber policeman" and collected quantitatively with a Pasteur pipette, using 3x3 ml. plate rinses of BSS, into conical glass centrifuge tubes. The cells were sedimented by centrifugation at 600 g. for 5 min. at 4^{o} and then washed with 2x10 ml. portions of ice-cold BSS before the pellet was drained by inversion. The pellet was then suspended in 0.5 ml. ice-cold 0.2 N perchloric acid and stored overnight at 4^{o}.

The suspension was centrifuged at 600 g. for 5 min. at $4^{o}$the following day and the supernatant was removed for cautious neutralisation to pH 7 with potassium hydroxide. The precipitate of potassium perchlorate which resulted was packed by centrifugation at 600 g. for 5 min. and the supernatant was removed. The supernatant volume was adjusted, where appropriate, and 100 µl aliquots were assayed for radioactivity in dioxane based scintillator.

Appropriate volumes, to contain at least 2.5×10^{3} d.p.m., of this acid-soluble cell extract were applied to Whatman No.1 chromatography paper for separation of thymidine and its phosphorylated derivatives in the solvent system originally described by Krebs and Hems (1953), but modified according to the recommendations of Keir and Smellie (1959). Thymidine and its 5'-mono-, -di- and -triphosphates (50 mµmoles each) were applied to each sample spot to be analysed, to act as markers. The markers were subsequently located under UV light. Equal areas around each spot were excised, cut into segments and dried in vials before 10 ml. toluene based scintillator fluid that contained Hyamine (5%) (b., above) was added and the radioactivity assayed as before.

5. Examination of DNA polymerase activity in vitro.

a. Cell cultivation procedures

Cells from stock were conditioned to grow in Eagle's medium (minimal essential medium, Burroughs Wellcome Ltd., England) supplemented with tryptose phosphate broth (10% v/v, III, B.1.c.iii) and foetal calf serum (10% v/v); the complete medium is hereafter referred to as ETS_{10}. Bulk cultivation was carried out according to the method of House and Wildy (1965) in rotating 2½ l. Winchester bottles charged with 200 ml. of this medium (ETS_{10}) and gassed with 5% CO_2 in air. The medium was changed according to acidity and the cells subcultured into fresh 2½ l. bottles at intervals, when the cells had reached confluence, using the standard procedures of trypsinisation (III, B.3.a.).

Cells were removed for extraction of DNA polymerase with a flat "wiper" blade applied firmly to the inner surface of the bottle as it was rotated. The cells were collected in medium (ETS_{10}), transferred to Universal containers and packed by centrifugation at 600 g. for 10 min.

b. Extraction of DNA polymerase from cells

The method outlined by Keir and Shepherd (1965) was followed. The cell pellet (a., above) was resuspended in 10 vols. 1 mM ethylenediaminetetracetic acid (EDTA) for homogenisation (ice-cooled) by a Potter-type homogeniser. Cell disruption was confirmed microscopically (1% crystal violet in 0.1% citric acid) before the suspension was centrifuged (Spinco Ultracentrifuge, Rotor 40, Model L; 105,000 g., 60 min.). The supernatant was collectively harvested and immediately made 0.02 M with 0.8 M tris-(hydroxymethyl)-methylamine (tris-Hcl) buffer, pH 7.5, and 0.15 M with solid potassium chloride (KCl) before storage at 0^{o}. This fraction was referred to as the cytoplasmic extract (CE) and was used for assay within one week of extraction.

Nuclear extract (NE) was prepared by resuspension of the pellet, obtained in the above preparation of CE, in buffer (0.02 M tris-HCl buffer, pH 7.5, 0.15 M KCl, 1 mM EDTA) and followed by sonication (Ultrasonic Power Unit, Measuring Scientific Equipment, England; 4x15 sec. bursts). Nuclear disruption was confirmed microscopically

(1% crystal violet in 0.1% citric acid) before ultracentrifugation of the sample (105,000 g. for 60 min., as above). The resulting supernatant (NE) was stored at 0°.

In the early experiments, 2-mercaptoethanol (5 mM final concentration) was included in all the buffers used in the enzyme preparation steps outlined above. However, for the reasons described in III, C.3.f., this was omitted latterly.

c. Estimation of protein in enzyme preparations

The protein content of the enzyme preparation was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951). Reagent A contained 2% (w/v) Na_2CO_3 in 0.1 N NaOH. Reagent B was freshly prepared with equal parts of aqueous solutions of 1% (w/v) $CuSO_4$, $5H_2O$ and 2% (w/v) sodium potassium tartrate. Reagent C was a mixture of reagent B (1 ml.) and reagent A (50 ml.). Reagent D consisted of dilute Folin Reagent (Folin-Ciocalteu phenol reagent titrated with sodium hydroxide to a phenolphthalein end point and diluted to 1.0 N with respect to acid).

A mixture of 1 ml. test solution and 5 ml. reagent C was allowed to stand for 10 min. before 0.5 ml. reagent D was added and the whole solution rapidly mixed. After at least thirty minutes, aliquots were compared against a reagent blank at 500 and 700 mμ on a Unicam SP 600 Spectrophotometer. A solution of bovine serum albumin (Armour Pharmaceuticals Ltd., Eastbourne, England) was used as standard.

d. Preparation of DNA

DNA was prepared from calf thymus according to the method of Kay, Simmons and Dounce (1952) and finally dissolved in 0.01 M sodium chloride. The concentration of DNA was estimated (Unicam SP 600) assuming $E^{1\ cm.}_{260\ m\mu} = 20$, with a solution of 1 mg. DNA/ml.

e. Assay of DNA polymerase activity

The method of Keir and Shepherd (1965) was followed. Aliquots of enzyme extract (CE or NE) were incubated at 37^{o} in a water bath in a solution (0.25 ml.) containing the following (µmoles per assay in brackets) potassium chloride (15); tris-HCl buffer, pH 7.5,(5); EDTA (0.1); $MgSO_4$ (2); deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, ^{32}P dTTP, 50 mµmoles each); calf thymus DNA, thermally denatured, 100 µg.

Samples (50 µl) were withdrawn at intervals during incubation for application to filter discs (Whatman, No.1, 1 inch, pretreated with bovine serum albumin (Armour, 50 µl (2 mg./ml.)) which were dropped into ice-cold 5% TCA (containing sodium orthophosphate $Na_4P_2O_7$, $10\ H_2O$ (B.D.H., Analar grade) 50 mM; 15 ml. per disc). Three further rinses in the same volume of ice-cold 5% TCA were followed by absolute ethanol (twice) and ether (twice) rinses. The dried filters were mounted on planchettes to be assayed for radioactivity (Nuclear Chicago Gas Flow Counter, fitted with a thin end window). The efficiency of counting by this technique was of the order of 50% for ^{32}P, with background counts less than one per minute.

Where possible the chemicals for addition to the assay system were dissolved and diluted in the same buffer as enzyme, i.e., tris-HCl, 0.02 M, pH 7.5, potassium chloride, 0.15 M. EDTA, 1 mM.

In the early experiments, 2-mercaptoethanol (5 mM final conc.) was included in the buffers used in the enzyme assay. However, for the reasons described in III, C.3.f., this was omitted latterly.

6. Treatment of DNA with MNNG in vitro.

The method of McCalla (1968a) was followed. Calf thymus DNA (Sigma Chemical Company, Missouri, USA) was dissolved by a gentle stirring, at 4^{o}, in 0.05 M sodium chloride - buffered at pH 7 with 0.01 M phosphate buffer (III, B.1.c.vii) - to give a final concentration of 2.6 mg./ml. Aliquots were made 2 mM with respect to MNNG (stock solution 20 mM in 0.01 M phosphate buffer, pH 6) with and without cysteine (2 mM final, fresh solution 20 mM in 0.05 M sodium chloride, pH 7) and incubated at 37^{o} for 3 hours.

Samples were placed in crushed ice, for 10 min., before absolute ethanol (two volumes) was layered over the solution. The DNA that precipitated at the interface was collected carefully by spooling on a glass rod, washed twice in 25 ml. absolute ethanol, and then redissolved in 0.05 M sodium chloride. The process of precipitation and ethanol washing was repeated before final dissolution of DNA in 0.01 M potassium chloride.

Comparison of extinction at 260 mμ (Unicam SP 600) given by samples before and after treatment showed a yield in control and MNNG exposed samples of approximately 60% and 50% of initial values, respectively. This suggested that the DNA was degraded, possibly via enzymic or spontaneous hydrolysis, during treatment at 37^{o}. The DNA samples were stored at 4^{o} until use.

Such treatment would be expected to give one alkylation per 10^{4} nucleotides for exposure with cysteine, with lower values for exposure to MNNG alone (McCalla, 1968b).

C. Results

1. Cell growth studies with Chinese hamster fibroblasts.

a. Cell growth rate and the effect of MNNG

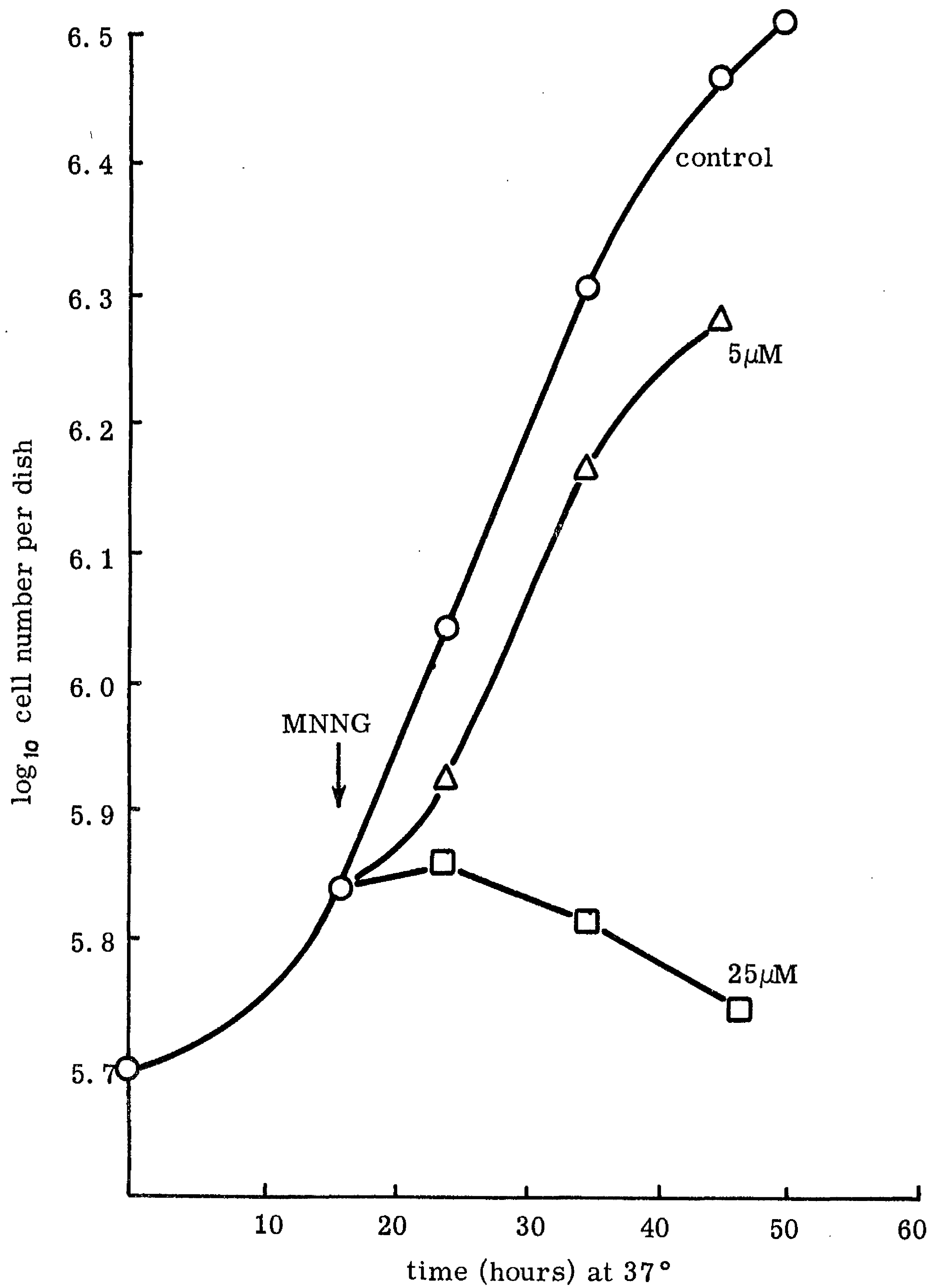
A measure of the rate of cell multiplication in monolayer cultures was obtained by the enumeration of cells harvested from individual dishes at intervals during incubation of cultures set up as described (III, B. 3.c.). The increase in cell number plotted logarithmically against time, during the exponential phase of growth, provided values of the population doubling time (i.e., mean cell generation time). A typical growth curve of the cells used in this study is shown in Fig. 17, from which a mean cell generation time of 12.5 hours was calculated. Values between 11.5 and 14 hrs. were obtained at other times during the course of the study. A mean cell generation time around 13 hrs. for this cell-line (Don) was in agreement with other workers (Ockey, Hsu and Richardson, 1968; Stubblefield, Klevecz and Deaven, 1967).

Population growth showed three phases (Fig. 17), namely, (a) lag phase, in which there was little increase in cell number, (b) exponential phase, when the increase was logarithmic, and (c) a phase of decreased growth rate, due to factors such as medium acidity or nutritional deficiency. It was found that an inoculum of 0.5×10^6 cells per dish (Esco, 60 mm.) gave rise to cultures in the (early) exponential phase of growth after overnight (16 hrs.) incubation. This number of cells was used routinely to provide cultures of approximately standardised maturity for MNNG exposure, during the early exponential phase. Entry of cultured cells into exponential growth phase was confirmed, by comparison of counts from control plates against a standard curve, before MNNG was added to the appropriate dishes.

Exposure to MNNG at a concentration of 25 µM completely inhibited growth (see Fig. 17), whilst at 5 µM the inhibition was temporary, with resumption after about 4 hours of growth at almost the normal rate. The combined results of two experiments to determine the growth inhibition at concentrations intermediate between 5 and 25 µM are shown in Fig. 18. It is evident that the effect on cell multiplication was approximately the

Figure 17. Inhibition of cell multiplication with MNNG.

Individual Petri dish cultures were set up as described in text. MNNG or control buffer was added to growth medium of the cells, in early logarithmic growth. At intervals afterwards the total cell number per dish was estimated according to standard procedures, after trypsinisation. The mean cell number on three dishes at each concentration is plotted on a logarithmic scale as a function of the incubation time.



6.5
6.4
6.3
6.2
6.1
6.0
5.9
5.8
5.7
$\log_{10}$ cell number per dish
control
5μM
25μM
MNNG
10
20
30
40
50
60
time (hours) at 37°

same at those intermediate doses and that removal of 20 μM MNNG medium after one hour and replacement with conditioned medium from control plates did not alter the inhibition. The similarity in response to short or prolonged exposure was in keeping with the known short half-life (90 min.) of the compound in the culture medium used (III, B.2.).

In the above experiments the cells were exposed to MNNG at a density of 1×10^6 cells per dish. A significantly different response was observed in cells exposed to MNNG at 1.5×10^6 cells per dish. Cells then showed a late recovery of multiplication after treatment, both for one hour and continuously, at 25 μM (see Fig. 18).

The study of cell growth in this form limited the observation to approximately two generation cycles. The growth rate of treated cells after subculture was not examined, however. Instead, the ability of treated cells to form colonies was used to assess the cytotoxicity of MNNG and to observe the morphological pattern shown by the treated cells.

b. Colony formation after MNNG exposure

When a comparison is made of the ability of cell populations of differing cell viability to produce colonies it is important to use optimum, standardised conditions. Cells which have been X-irradiated with a dose that prevents division will, as originally advocated by Puck and Marcus (1955), 'condition' the medium prior to inoculation with 'viable' cells, and provide optimum circumstances for colony formation. Using this 'feeder' layer technique, as outlined in III, B.3.g., plating efficiencies of more than 25% were regularly obtained with control-treated cells. The decrease in survival, as judged by colony formation, among cells exposed in the exponential phase of growth to different concentrations of MNNG is shown in the dose-response curve of Fig. 19. The concentration which reduced survivors by 50% (ID_{50}) of control was 15 μM for cells exposed at 1×10^6 cells per dish, whereas for cells exposed at 1.5×10^6 cells per dish the ID_{50} increased to 25 μM. This observation was in agreement with inhibition of cell multiplication rate noted

Figure 18. Inhibition of cell multiplication with MNNG.

Individual Petri dish cultures were set up as described in text. After overnight growth, MNNG or control buffer was added to growth medium in dishes (multiples) to give the stated concentration in μM. At intervals afterwards the cell number per dish was estimated according to standard procedures, after trypsinisation. The mean cell number on three dishes at each concentration is plotted on a logarithmic scale as the proportional increase above the number on control dishes, at the time of additions, as a function of incubation time.

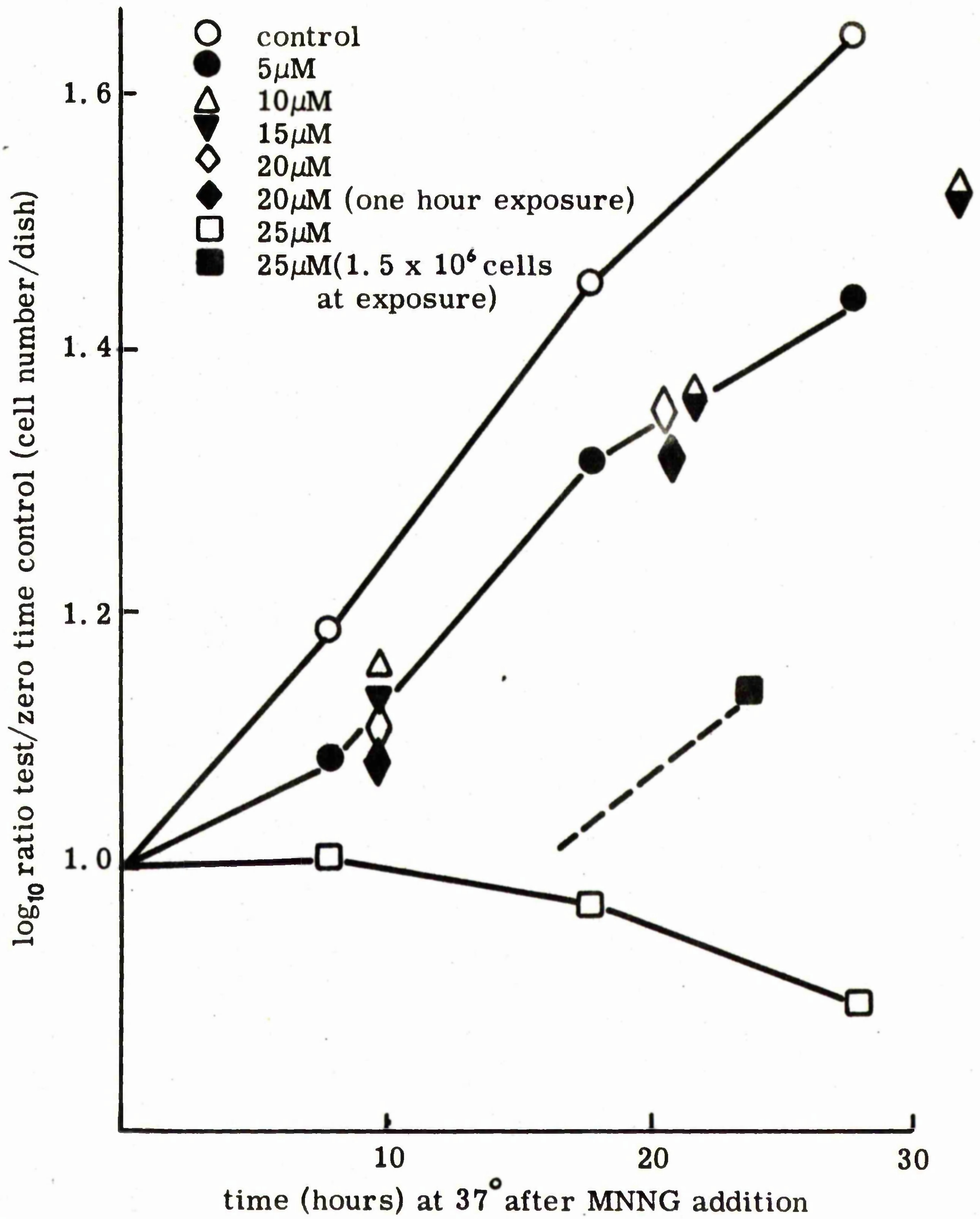
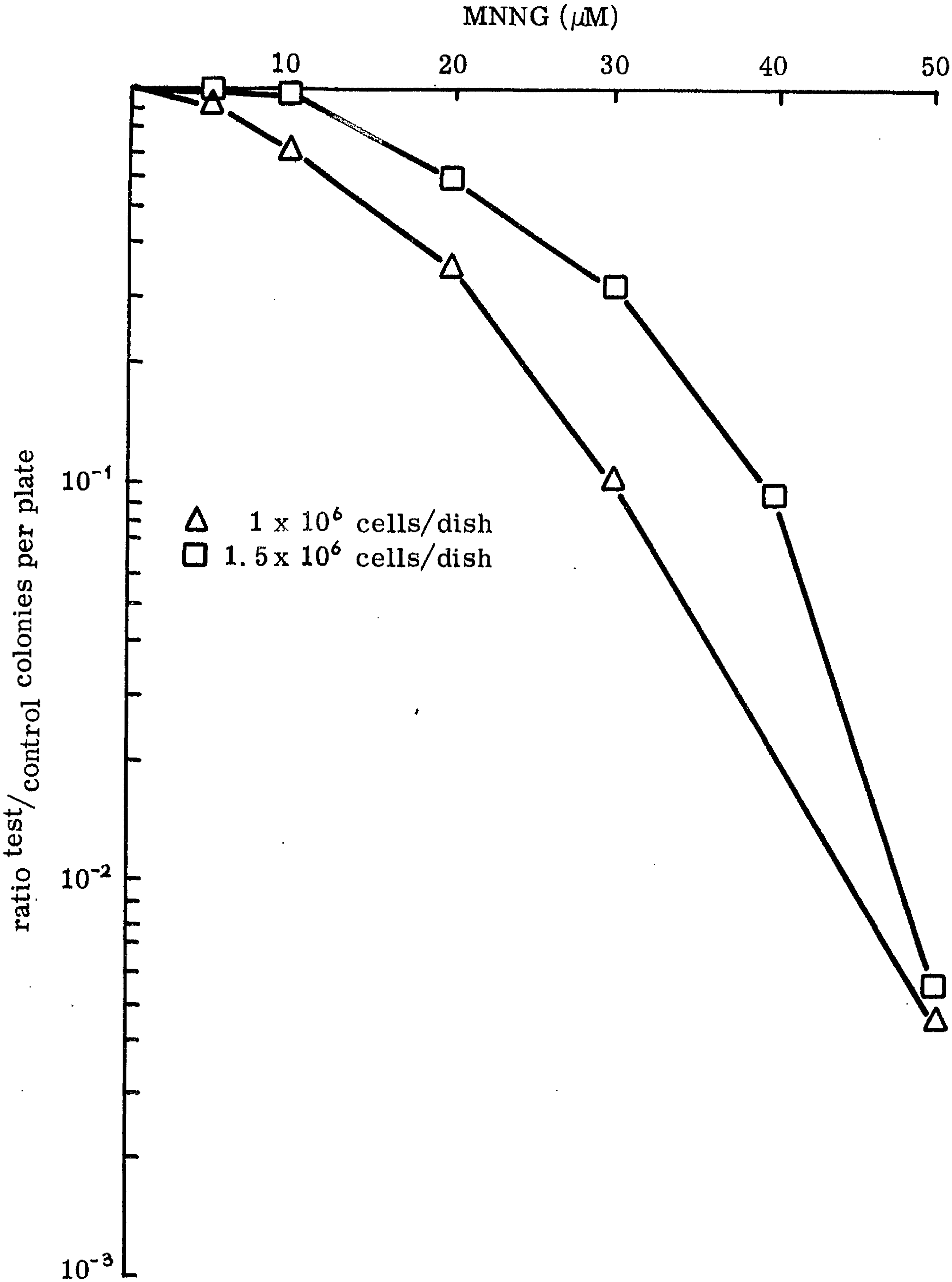

control
5μM
10μM
15μM
20μM
20μM (one hour exposure)
25μM
25μM(1.5 x 10⁶ cells
at exposure)
1.6
1.4
1.2
1.0
log10 ratio test/zero time control (cell number/dish)
10
20
30
time (hours) at 37° after MNNG addition

Figure 19. Survival of cells exposed to MNNG.

MNNG or control buffer was added to the medium of Petri dish cultures in logarithmic growth. After one hour incubation at 37^{o} the cells were harvested to be plated on irradiated feeder layers as described in the text. The number of colonies per dish (mean of at least three) at 9 days is plotted on a logarithmic scale as the ratio of the number of colonies on controls, against the initial concentration of MNNG in the medium.


MNNG (μM)
10
20
30
40
50
ratio test/control colonies per plate
10⁻¹
10⁻²
10⁻³
1 x 10⁶ cells/dish
1. 5 x 10⁶ cells/dish

above (a.) for populations exposed at different cell densities per dish.

The morphology of colonies derived from MNNG-treated and control-treated cells was examined carefully and compared. The colonies could be readily grouped, according to cellular orientation, as follows:-

(i) centrally compact and peripherally orientated (some colonies with scalloped edges) (see Fig. 20),

(ii) centrally compact and peripherally disorientated (see Fig. 21a),

(iii) dispersed and disorientated, shrunken cells.

The distribution of the different colony types on the dishes at nine days of incubation is shown on Table 13. The striking feature about the colonies of type (ii), which were observed only on dishes of MNNG treated cells, was their resemblance to the appearances reported as "neoplastic transformation" by other workers (Berwald and Sachs, 1963; Sanders and Burford, 1967). However, it was also observed that if the dishes of MNNG-treated cells, from the same experiment, were incubated for eleven days before preparation for examination, then the distribution of colony types was altered from that at nine days and approximated closely to the distribution on control plates (see Table 13). One factor which accounted for this difference at eleven days was the evidence of peripheral orientation in a proportion of colonies which formerly belonged to type (ii) (see Fig. 21b). This peripheral disorientation of type (ii) colonies, and possibly also the appearances of type (iii) colonies, may have been the result of delayed maturity of colonies, related to the inhibitory effects of MNNG. The reduced number of such colonies after further incubation was taken as an indication of recovery from these inhibitory effects. The colony size shown by MNNG-treated cells was generally smaller than controls at the same stage of incubation, but there were no colonies on any plates with the totally random and multilayered structure reported following the treatment of cells with the carcinogen, N-methyl-N-nitrosourea (Sanders and Burford, 1967).

The growth of treated cells subcultured from colonies was not examined. Experiments of animal inoculation with cells was not carried out.

Treatment	Days at 37^{o}	Distribution of colony types (% of total)		
		(i)	(ii)	(iii)
Control	9	95	0	5
20 µM MNNG (1 hr.)	9	60	15	25
20 µM MNNG (1 hr.)	11	90	5	5

Table 13. Distribution of colony types of MNNG-treated cells.

The different types of colonies, (i), (ii) and (iii), shown by cells plated according to procedures outlined in the text, are described in the text. At least 100 colonies were examined for an assessment of each treatment after the stated period of incubation. The significance of the change in distribution with increased incubation time, shown by MNNG-treated cells, is discussed in the text.

Figure 20. Colony formation of hamster fibroblasts (Don): normal.

The characteristic parallel orientation, and the radial appearance at the periphery, is shown by cells of a control colony at 9 days of incubation (magnification x 15).

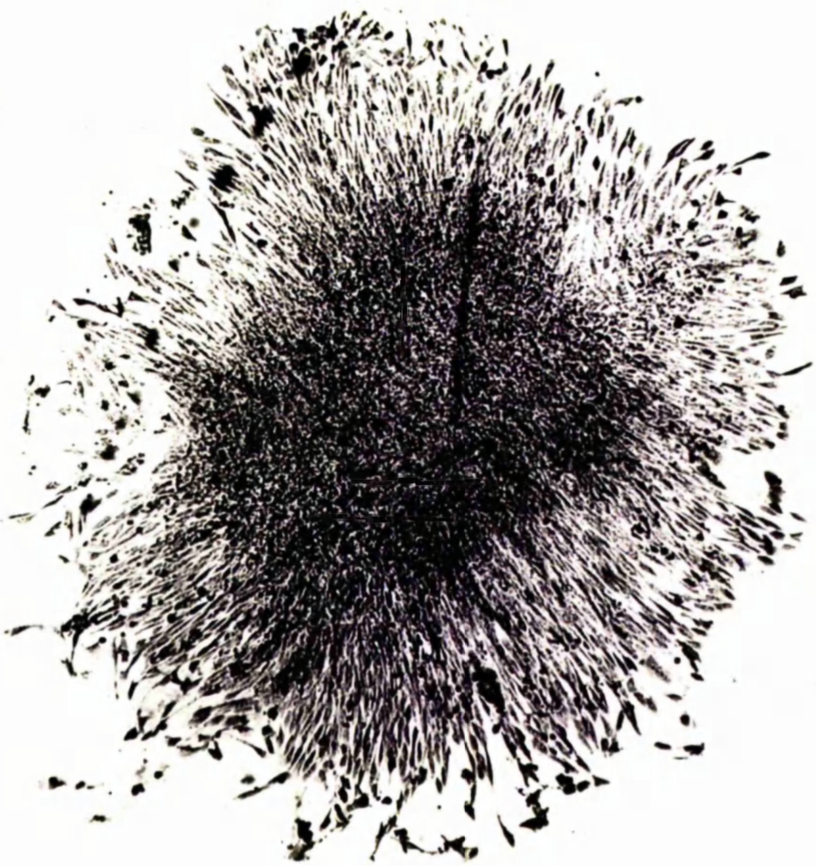


Figure 21.

Colony formation of hamster fibroblast (Don): MNNG treated.

(a) Appearance of colonies (type(ii),Table 13) derived from cells exposed for one hour to MNNG (20 µM, initial) as described in text, at 9 days of incubation. Note peripheral disorientation of cellular arrangement (magnification x 15).

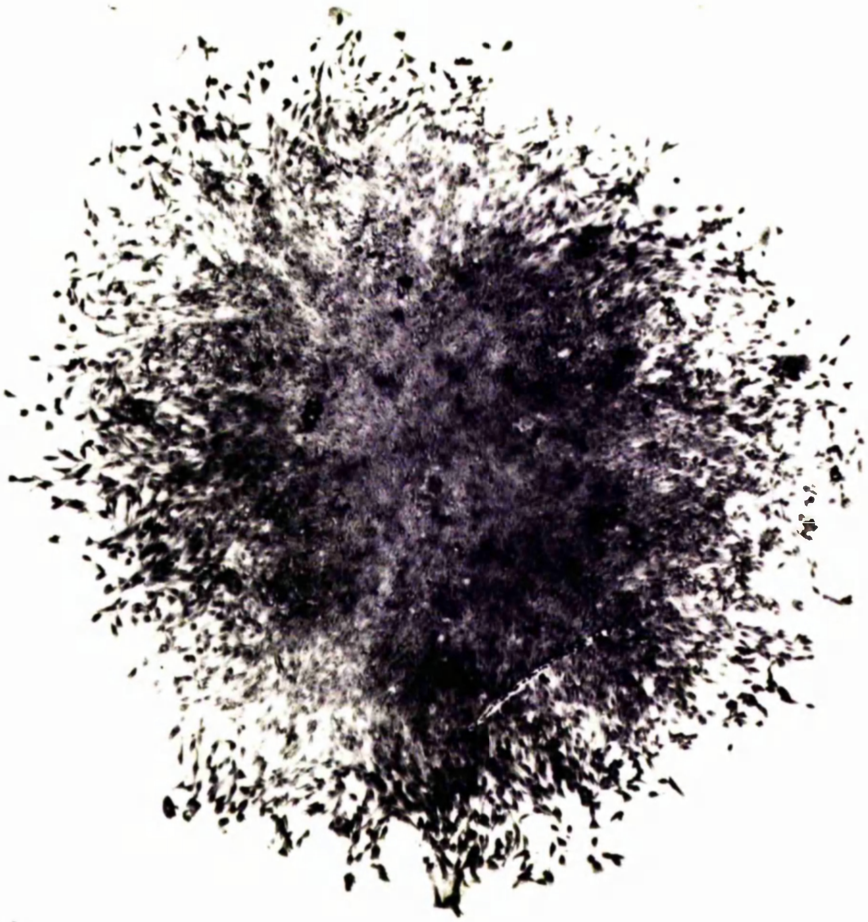
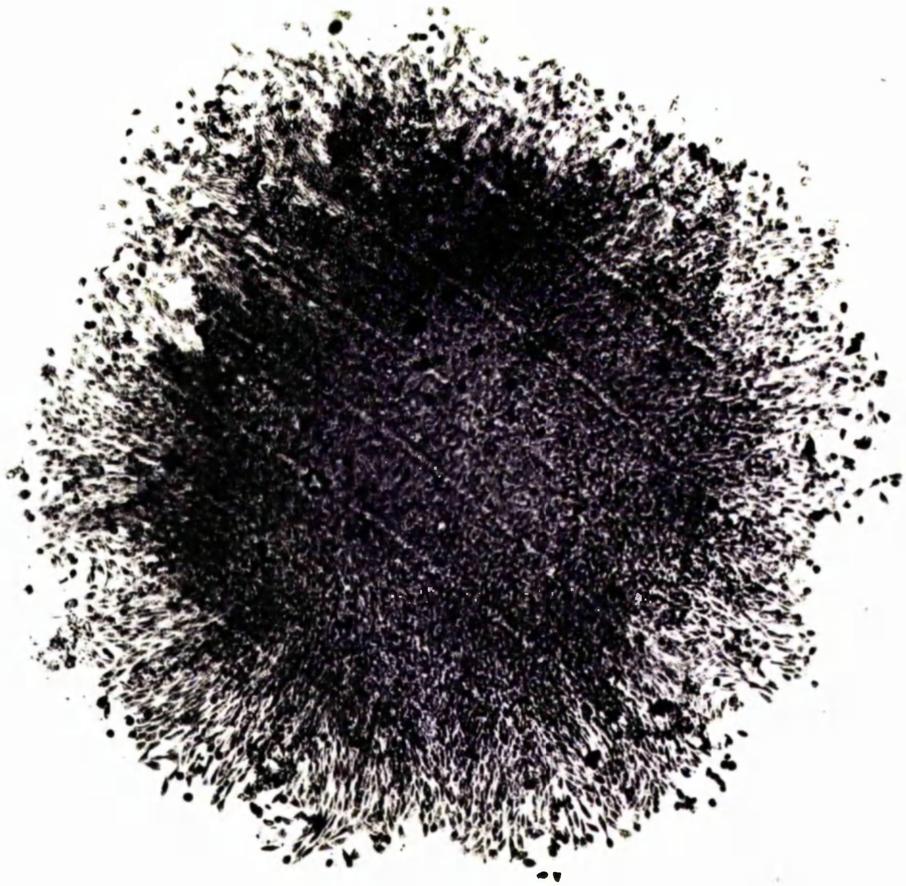


Figure 21.

Colony formation of hamster fibroblast (Don): MNNG treated.

(b) Appearance of colony, similar to (a), above, derived from cells exposed for one hour to MNNG (20 μM, initial), as described in text, at 11 days of incubation. Note tendency to parallel orientation of cellular arrangement at the periphery (magnification x 15).



c. Life cycle of cells and sensitivity to MNNG

(i) Accumulation of cells at metaphase with Colcemid. The numerical analysis of the rate of accumulation of cells in logarithmic growth arrested at a recognisable stage in the growth cycle has been used (Puck and Steffen, 1963) to determine the duration of the different phases of the life cycle in cultured mammalian cells. Suitable modification to the analysis has permitted localisation of action of the metabolic inhibitors, puromycin and actinomycin D to specific periods in the G_2 phase of the cycle (Tobey, Peterson, Anderson and Puck, 1966). Since the measurement of growth rate and colony formation of cells exposed to MNNG served as an indication of the gross cytotoxic effect only, experiments were carried out as outlined in III, B.3.e. Accumulation of cells, at metaphase in mitosis, with Colcemid, which interferes with the formation of the microtubules of the centriole complex in the mitotic apparatus (Borisy and Taylor, 1967; Stubblefield and Brinkley, 1967) was attempted. The results of a typical experiment are shown in Fig. 22a. Failure to accumulate metaphase cells in a linear fashion, essential for the success of the analysis, persisted in spite of alteration to technique involving concentration of Colcemid, and procedures of cell inoculation, collection and fixation. The ability of the hamster cells to escape from arrest by Colcemid was observed, however. This finding was confirmed in reports and correspondence with other workers (Stubblefield _et al_., 1967; Engelberg, personal communication, 1968). For this reason further experiments of this nature were not performed.

(ii) Assessment of mitotic index after MNNG exposure. The proportion of cells undergoing mitosis (prophase, metaphase, anaphase and early telophase), the mitotic index, remains constant in a population of cells in exponential growth. Alteration to the mitotic index in cells exposed to X-irradiation has been used by Watanabe and Okada (1966) to demonstrate sensitivity at a point in G_2 phase

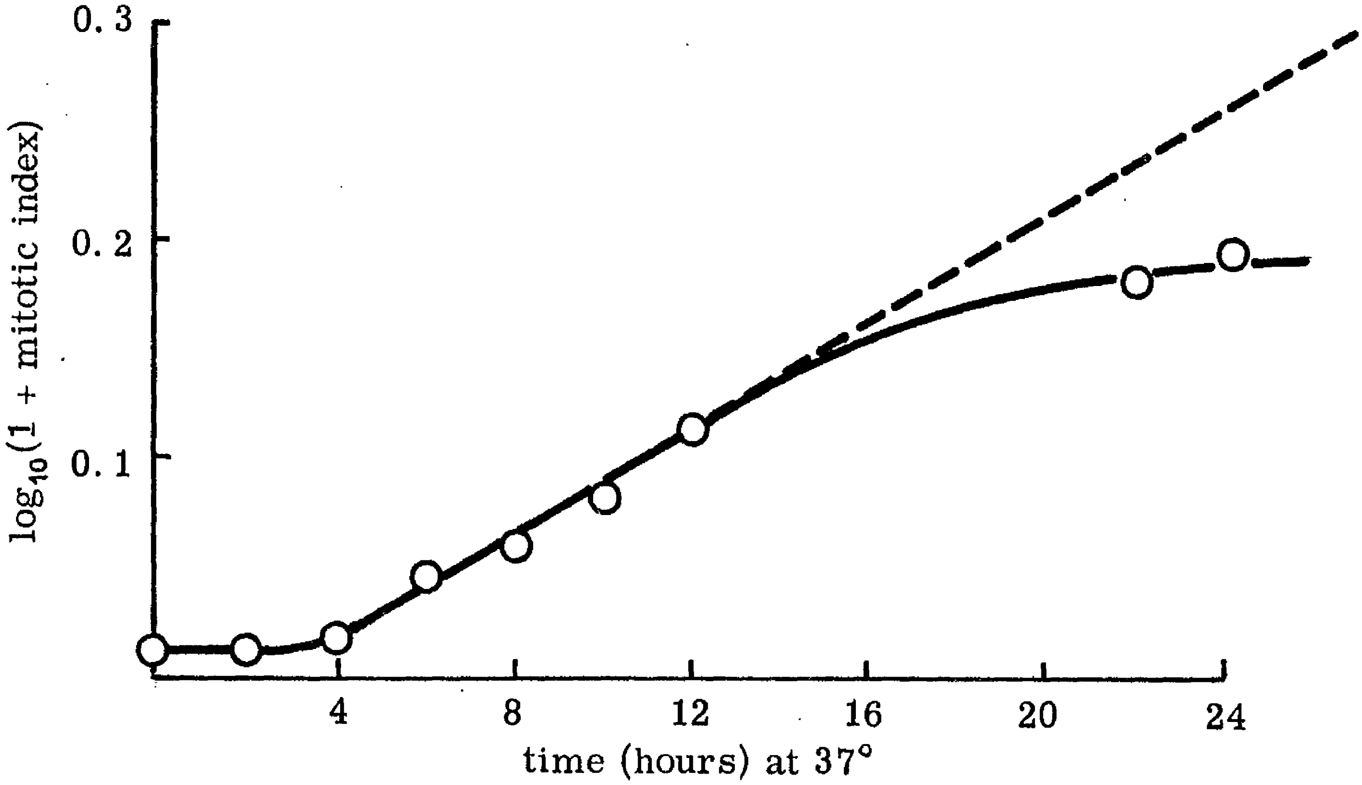
Figure 22.

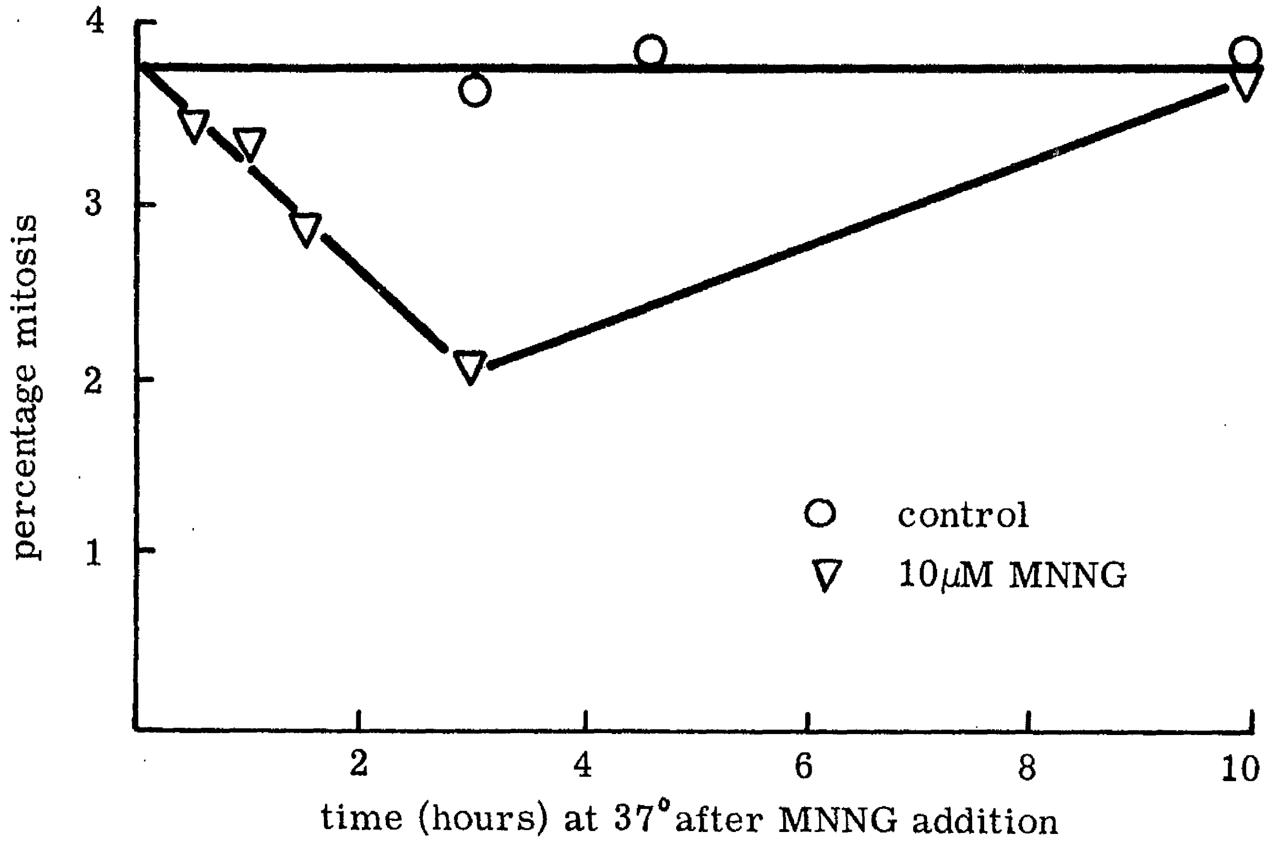
(a) Rate of accumulation of cells at metaphase.

The mitotic index, N(M), was assessed in cultures at intervals following addition of Colcemid to the growth medium, according to procedures outlined in the text. A total number of at least 1000 cells was assessed for each time interval. The value log (1 + N(M)) is plotted as a function of the time interval after Colcemid addition. The interpretation of results is described in the text.

(b) Mitotic index in cell cultures after MNNG exposure.

The mitotic index was assessed in cultures after addition of MNNG (10 μM final) or control buffer to growth medium of cells in logarithmic growth phase, according to procedures outlined in text. A total of at least 1000 cells was examined for each point. The mitotic index (percentage of total cells) is plotted as a function of time after MNNG was added to the medium, for comparison against control-treated cells. The interpretation of results is described in the text.


0. 3
0. 2
0. 1
log10(1 + mitotic index)
4
8
12
16
20
24
time (hours) at 37°


4
3
2
1
percentage mitosis
2
4
6
8
10
time (hours) at 37° after MNNG addition
control
10μM MNNG

of the cell division cycle of cultured cells.

The mitotic index in a population of cells exposed to 10 μM MNNG at 1×10^6 cells per dish is shown in Fig. 22b. The alteration to mitotic index, although not corrected for cell viability, indicated the sensitivity to MNNG of the G_2 phase of the cell cycle. This was deduced from the immediate decrease in mitotic index after MNNG addition to the medium, thus indicating a failure of cells in G_2 to enter into mitosis.

2. Studies with radioactive isotopes of metabolic inhibition in Chinese hamster fibroblasts.

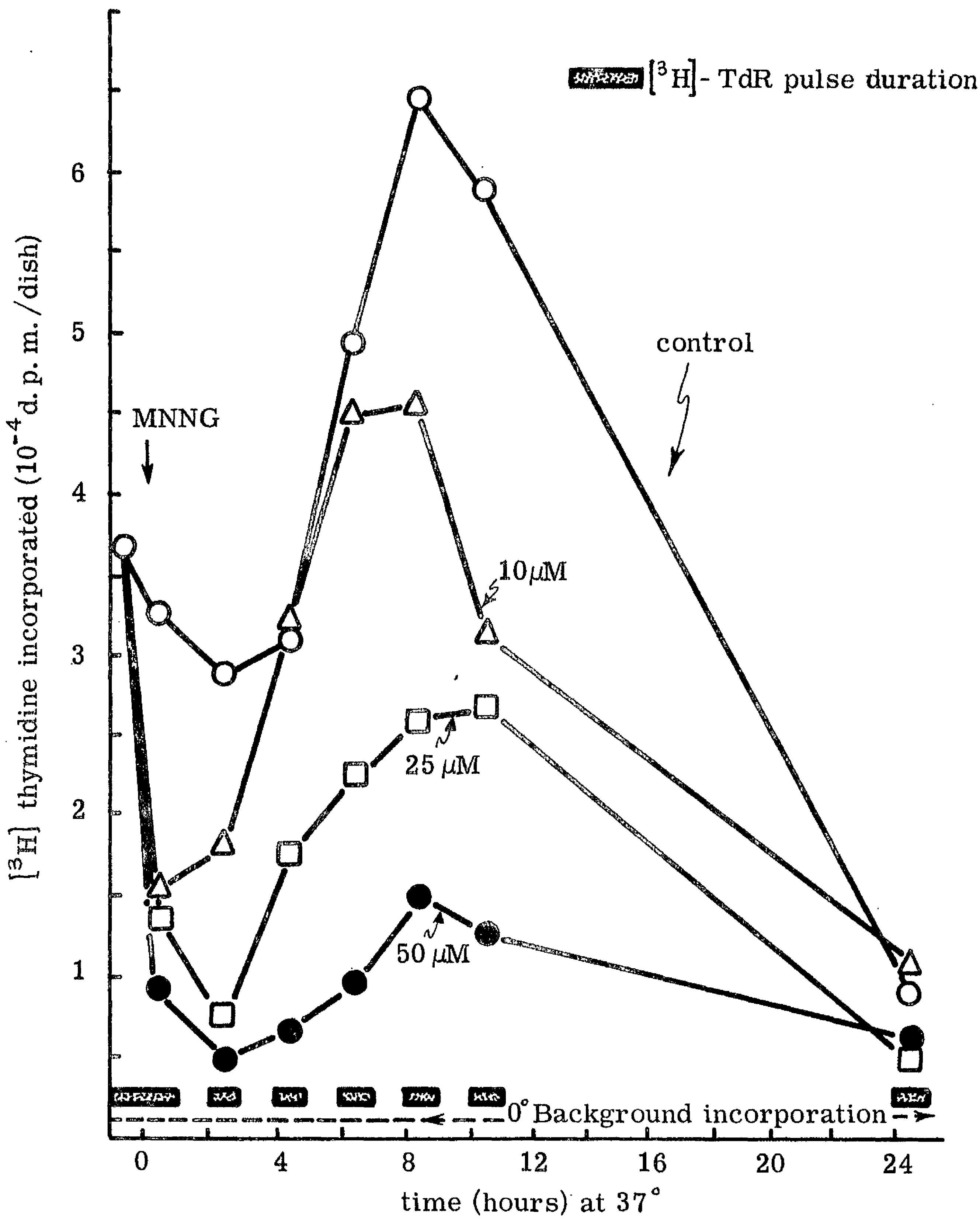
a. Incorporation of $[^3H]$thymidine into DNA

(i) Incorporation of $[^3H]$thymidine during the 24 hour period after exposure to MNNG. Cell cultures, set up according to III, B.3.c., were examined in exponential growth for their ability to incorporate $[^3H]$thymidine into DNA at intervals after addition of MNNG to the growth medium. The results of a typical experiment, in which the cells were exposed at a density of 1.3×10^6 cells per dish, are shown in Fig. 23. There was an inhibition of incorporation in control and treated populations that was followed by recovery; the extent of the former appeared to be directly related to the dose of MNNG whilst the latter showed an inverse relationship.

The explanation of the immediate decrease in incorporation of isotope by controls was believed to be the decrease in temperature to which the cells were exposed during the technical procedures involved in the simultaneous addition, to the replicated cultures, of MNNG and radioactive isotope. Rao and Engelberg (1966) have shown that cultured mammalian cells in G_1 phase of growth are most sensitive to a decrease in temperature, which inhibits their entry into the next phase, S, when DNA is synthesised. Thus, a failure of cells to enter S phase, whilst cells in late S phase progress into G_2 phase, would result in a decrease in $[^3H]$thymidine incor-

Figure 23. Effect of MNNG on $[^3H]$ thymidine incorporation into DNA of cells.

MNNG or control buffer was added to the medium of Petri dish cultures in logarithmic growth phase to give the indicated final concentration, according to procedures described in the text. At intervals afterwards individual Petri dishes cultures were exposed to $[^3H]$ thymidine (10 μcuries/dish) for one hour, then prepared for assessment of radioactivity incorporated into DNA, according to standard techniques. The total amount of radioactivity per dish (mean of three) is plotted as a function of the time interval after MNNG (or control) addition to the medium. The inhibition and recovery of incorporation in control- or MNNG-treated cells is interpreted in the text.


[3H]- TdR pulse duration
[3H] thymidine incorporated (10-4 d. p. m. /dish)
6
5
4
3
2
1
MNNG
control
10μM
25 μM
50 μM
0° Background incorporation
0
4
8
12
16
20
24
time (hours) at 37°

poration by the cell cultures.

The inhibition observed in MNNG-treated cells was much greater. The extent of inhibition and failure to regain normal rates of incorporation after exposure to 25 μM MNNG or more was in agreement with earlier experience with MNNG as a growth inhibitor (III, C.1.a.). The inability to maintain normal rates of incorporation beyond 7 hr. after exposure was taken as evidence that the cells were not re-entering S phase of the cycle following division. Furthermore, the relationship of this inhibition to the concentration of MNNG was examined more closely in later experiments when any contribution to the inhibition by the 'temperature' effect, noted above, was avoided (iii, below).

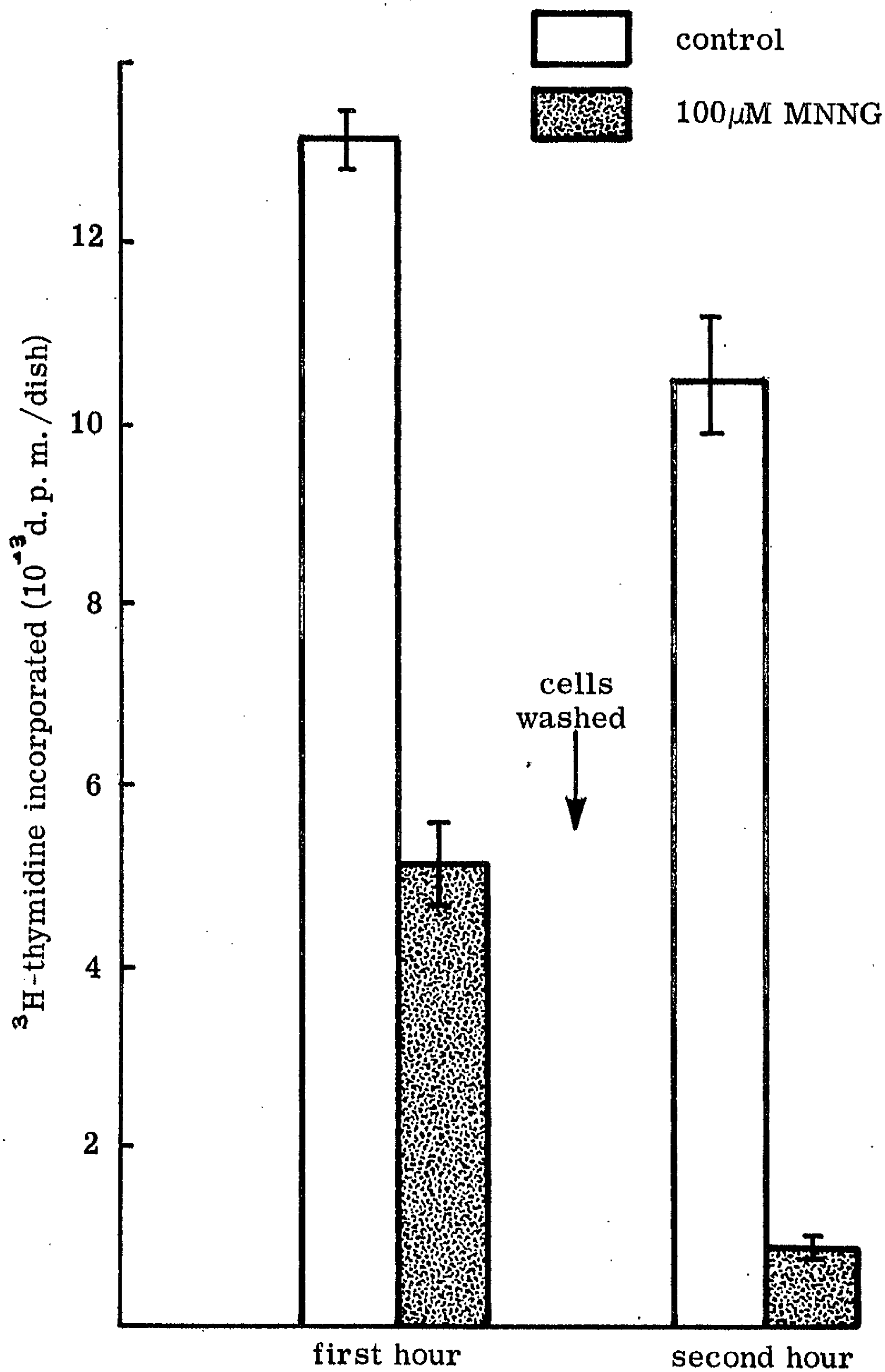
At 24 hr. the incorporation by controls and MNNG-treated cells was considerably reduced below zero time values, although still above background incorporation. Factors such as medium acidity and exhaustion of nutriments now contributed to the other effects causing decreased incorporation of $[^3H]$thymidine into DNA and limited the useful observations in these cultures to the first 10 hr., after exposure.

(ii) Reversal of inhibition of $[^3H]$thymidine incorporation. The inhibition of DNA synthesis by hydroxyurea is readily reversed by removal of the drug (Adams and Lindsay, 1967). Fig. 24 shows the results of an experiment in which the cultured cells were washed with Saline D2 after exposure to MNNG for one hour. The medium was replaced with conditioned medium from untreated controls and the amount of $[^3H]$thymidine incorporated by these cells in the following hour (second after MNNG exposure) was assayed according to standard procedures (III, B.4.c.i.). The results were then compared with those from cells of the same experiment exposed to $[^3H]$thymidine for the first hour of MNNG treatment, and also with those of controls.

It is evident from Fig. 24 that the inhibition by MNNG is not immediately reversed by removal of the drug.

Figure 24. MNNG inhibition of $[^3H]$ thymidine incorporation into DNA of cells: failure to reverse by washing.

MNNG or control buffer was added to the medium of Petri dish cultures in logarithmic growth phase. One set of dishes (in triplicate) was immediately exposed to $[^3H]$ thymidine (10 μcuries/dish) for one hour, then the cells were prepared for assessment of radioactivity incorporated into DNA, according to standard techniques. The medium of other dishes was replaced after the first hour with medium from untreated cell cultures, as described in the text, and the cells were then exposed to $[^3H]$ thymidine (10 μcuries/dish) for one hour before preparation as above for radioactive assessment. The total amount of radioactivity incorporated per dish (mean of three) of control- or MNNG-treated cells is shown before and after washing procedure.



control
100μM MNNG
^{3}H-thymidine incorporated (10^{-3} d. p. m./dish)
12
10
8
6
4
2
cells washed
first hour
second hour

(iii) Incorporation of $[^3H]$thymidine into DNA during the first hour after exposure to MNNG. The relationship, during the first hour after exposure, between the concentration of MNNG and the extent of inhibition of $[^3H]$thymidine incorporation was studied over a wider range of MNNG concentration than before (a.i., above). At this time (first hour) there was no evidence of recovery. In these experiments the influence of temperature decrease (a.i., above) was avoided in the following manner. Cell cultures were set up as before (III, B.3.c.), but were incubated, contained in air-tight boxes, gassed with 5% CO_2 in air, in a warm room maintained at $37^{\circ} \pm 0.5$. All subsequent procedures were carried out in the warm room.

The combined results of two experiments are shown in Fig. 25, from which it is evident that an exponential effect, which extrapolates below zero concentration, was obtained with respect to MNNG concentration. Although contributory factors, such as alteration to medium pH on brief exposure to air, and/or, disturbances of local conditions around cells caused by handling procedures, cannot be excluded, the result was highly suggestive of a single intracellular target for MNNG, with respect to the inhibition of $[^3H]$thymidine incorporation.

However, before examination of the individual enzyme pathways involved in DNA synthesis was undertaken, to further examine this inhibition of $[^3H]$thymidine incorporation by MNNG, experiments were carried out with other radioactive nucleic acid precursors, and with radioactive amino acids, to determine effects of MNNG on RNA and protein synthesis.

b. Incorporation of $[^3H]$deoxyadenosine into DNA and RNA

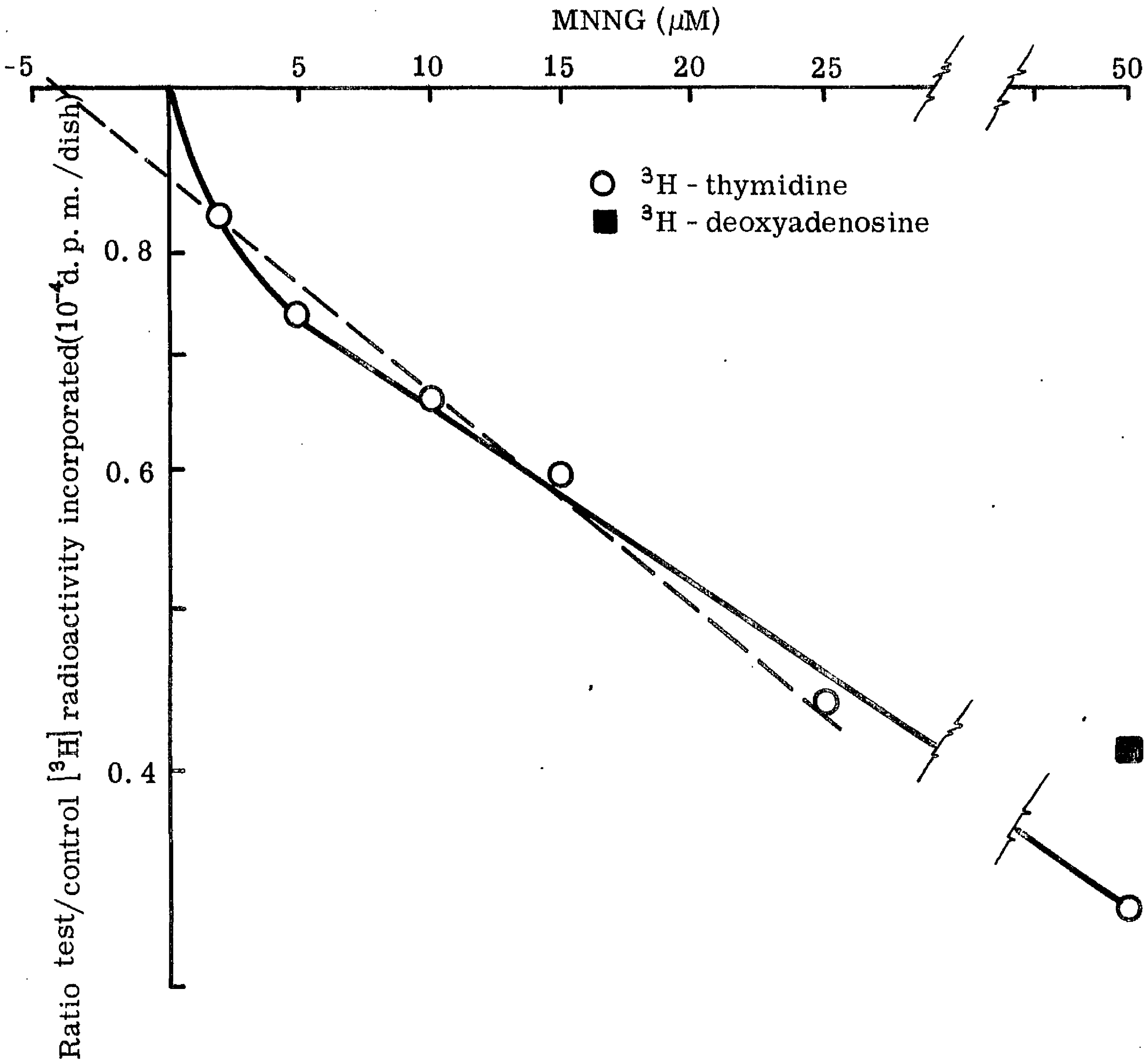
Experiments with $[^3H]$thymidine (a., above) demonstrated inhibition of precursor incorporation into DNA by MNNG. To determine whether this effect was apparent also with purine deoxyribonucleoside precursors of DNA the experiment was repeated according to III, B.3.c.i., and the cells

Figure 25. MNNG inhibition of $[^3H]$ thymidine incorporation into DNA of cells: first hour.

MNNG or control buffer was added to the medium of Petri dish cultures in logarithmic growth phase and the cells immediately exposed to $[^3H]$ thymidine (10 μcuries/plate) for one hour. The cells were then prepared for assessment of radioactivity incorporated into DNA, according to standard techniques. The total radioactivity incorporated per dish (mean of at least three) is plotted on a logarithmic scale, as the ratio of control incorporation, according to the initial concentration of MNNG in the medium. The inhibition is shown both as a biphasic response (solid line), and as a linear response which extrapolates below zero dose (dotted line); the interpretation of the data is discussed in the text.

The inhibition of $[^3H]$ deoxyadenosine incorporation into DNA, from the values given in Table 14, is plotted similarly for comparison.

The technique of cell cultivation, to avoid exposure to decreased temperatures during manipulation procedures, is described in the text.



MNNG (μM)
-5
5
10
15
20
25
50
³H - thymidine
³H - deoxyadenosine
0.8
0.6
0.4
Ratio test/control [³H] radioactivity incorporated(10⁻⁴d. p. m. /dish)

'pulsed' with $[^3H]$deoxyadenosine during the first hour after MNNG addition to the medium. The harvested cells were treated appropriately (III, B.4.c.iii.) to give an assessment of the amount of $[^3H]$ radioactivity incorporated into DNA and RNA.

The extent of incorporation of radioactive isotope by MNNG treated cells, expressed as a percentage of incorporation into control cells, is shown in Table 14. At both concentrations the inhibition was greater for DNA than for RNA. The greater total quantity of radioactivity was always present in RNA, however. The experiment also established that for both thymidine and deoxyadenosine, as representatives of pyrimidine and purine deoxyribonucleosides, respectively, the inhibition was of the same order of magnitude (see Fig. 25).

c. Incorporation of $[^3H]$uridine into RNA during the 24 hour period after treatment with MNNG

Pilot studies established that under the experimental conditions less than 2% radioactivity was incorporated into DNA. This figure was within the limits of the experimental error; therefore no correction was made for this loss of specificity of isotope incorporation. The inhibition of incorporation of $[^3H]$uridine into RNA after MNNG treatment is shown in Fig. 26 and clearly demonstrates a difference in response to that observed for the incorporation of $[^3H]$thymidine into DNA (Fig. 23). Although the pattern of immediate inhibition and recovery was similar the same relationship of inhibition to concentration of MNNG was not apparent.

Furthermore, the rate of incorporation of precursor into RNA, at 5 hr. or more after treatment with 70 μM MNNG, was increased above controls (see Fig. 26). Here the incorporation by treated cells can be compared with incorporation by controls at zero time, since the growth of the MNNG treated populations would be arrested (III, C.1.a.).

d. Incorporation of $[^{14}C]$amino acids after exposure to MNNG

Preliminary experiments were made to determine the amount of

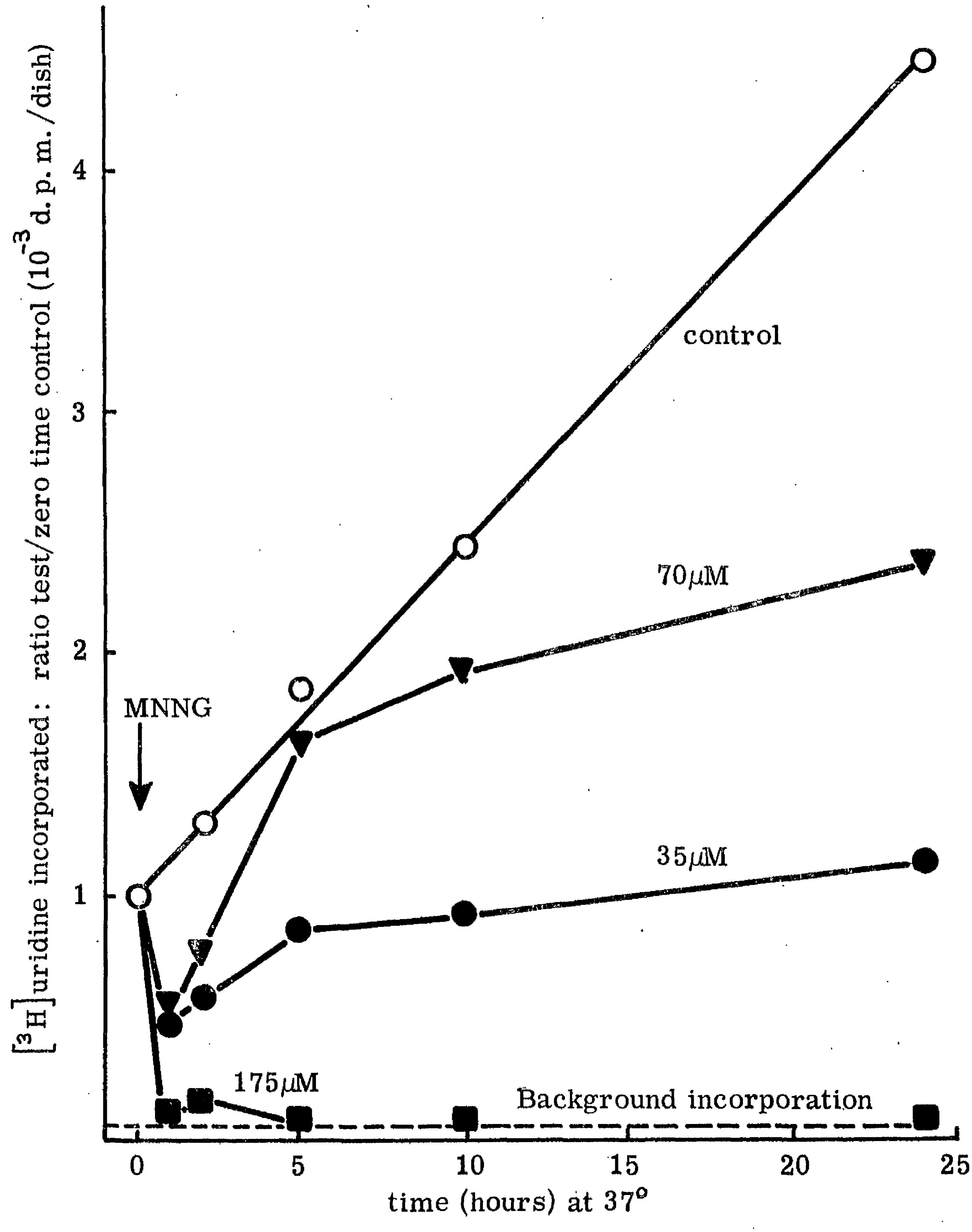
Table 14. Effect of MNNG on $[^3H]$ deoxyadenosine incorporation into DNA and RNA of cells

MNNG or control buffer was added to the medium of Petri dish cultures, in logarithmic growth phase, to give the indicated final concentration, according to procedures outlined in the text. The cells were immediately exposed to $[^3H]$ deoxyadenosine (10 μcuries/plate) for one hour and then harvested for preparation as described in the text. Assessment of radioactivity incorporated into DNA and RNA was carried out according to standard procedures. Results are given as the amount of radioactivity per dish (mean of four for each treatment). The radioactivity is shown as the total per dish, with the distribution in DNA and RNA and the percentage inhibition of incorporation at the concentration of MNNG. The percentage of the total radioactivity in DNA and in RNA at each treatment is also calculated.

	μM MNNG ⟶	0	50	100
Total	10^{-4} d.p.m./dish	58.53	38.69	15.14
DNA	10^{-4} d.p.m./dish	4.1	1.69	0.62
	% $^{test}/_{control}$	100	41	15
	% total d.p.m.	7.6	4.4	3.2
RNA	10^{-4} d.p.m./dish	54.4	37.0	14.52
	% $^{test}/_{control}$	100	68	27
	% total d.p.m.	92.4	95.6	96.8

Figure 26. Effect of MNNG on incorporation of $[^3H]$ uridine into RNA of cells.

MNNG or control buffer was added to the medium of multiple numbers of individual Petri dish cultures of cells in logarithmic growth phase, to give the appropriate final concentration, according to procedures outlined in text. At intervals afterwards, the cells were exposed to $[^3H]$ uridine (2.5 μcuries/plate) for 20 min. and prepared for assessment of radioactivity incorporated into RNA as described in text. The total amount of radioactivity per dish (mean of three) at each concentration (initial) of MNNG is plotted as the ratio of control incorporation, at the time of MNNG additions, against the time interval after MNNG was added to the medium. The inhibition of incorporation is shown for comparison with the linear increase in incorporation of radioactivity by control-treated populations. The significance of the observations is discussed in the text.



[3H]uridine incorporated: ratio test/zero time control (10-3 d. p. m. /dish)
4
3
2
1
0
5
10
15
20
25
time (hours) at 37°
control
70µM
35µM
175µM
MNNG
Background incorporation

radioactivity bound to tRNA molecules in the acid precipitable material extracted from cultured cells. No significant difference was detected between samples (a) untreated or (b) pre-incubated in 5% TCA at 65^{o} for 30 minutes to hydrolyse amino acid bound to tRNA. This step was, therefore, omitted from subsequent experiments.

The amount of $[^{14}C]$ radioactivity incorporated into acid precipitable material extracted from replicated cell cultures following exposure to MNNG is shown in Fig. 27.

e. The incorporation of $[^{3}H]$thymidine into phosphorylated thymidine derivatives after exposure of cells to MNNG

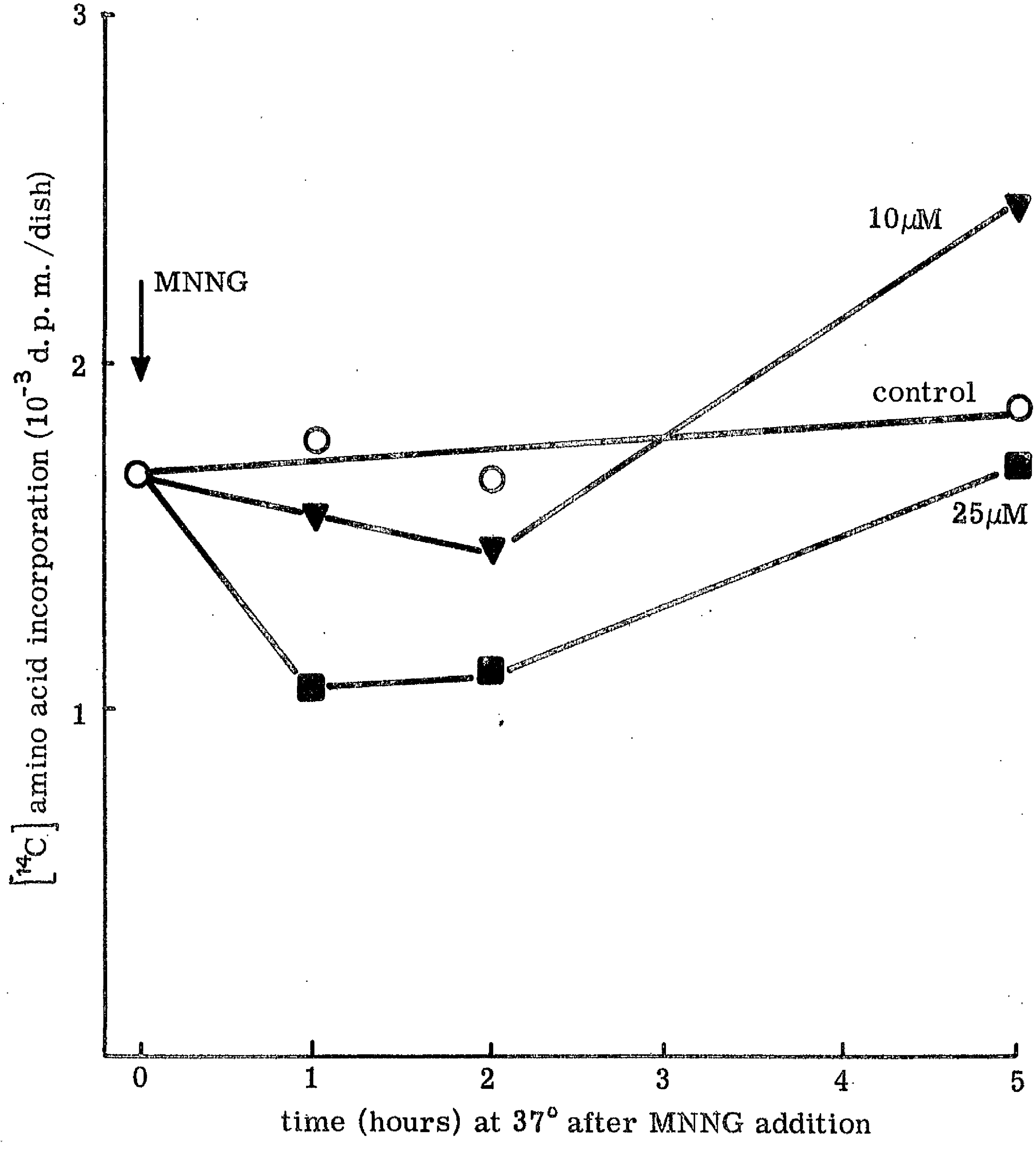
Acid-soluble extracts were prepared from control and MNNG treated cell cultures after exposure to $[^{3}H]$thymidine for the second hour following MNNG addition to the growth medium, and aliquots of the extracts were analysed by chromatographic procedures, all according to III, B.4.c.v. The R_f values for thymidine and the thymidine 5'-nucleotides observed in these experiments are given in Table 15, for comparison with values observed by Keir and Smellie (1959).

The proportion of radioactivity present as thymidine 5'-mono-, -di- and -triphosphate in acid-soluble extracts of control and treated cells is shown diagrammatically in Fig. 28. There was a slight decrease in the total amount of radioactivity extracted from cells treated with 20 µM MNNG but there was no significant impairment of phosphorylation of ^{3}H thymidine at either 20 or 100µM MNNG, compared to control.

Acid-precipitable radioactivity in the same cells, from which acid-soluble extracts had been taken after exposure to 20 and 100 µM MNNG, was assayed in the usual way (III, B.4.c.i.) and gave values 58% and 28% of control, respectively. This confirmed that inhibition of incorporation of $[^{3}H]$thymidine into DNA had occurred to the same extent as observed previously (III, C.2.a.iii, see Fig. 25).

Figure 27. Effect of MNNG on incorporation of $[^{14}C]$ amino acids into protein of cells.

MNNG or control buffer was added to the growth medium of individual Petri dish cultures of cells in logarithmic phase of growth, to give the appropriate final concentration, according to procedures outlined in the text. At the times indicated the cells were exposed to $[^{14}C]$ amino acids mixture (0.5 μcuries/dish) for 30 min. and then prepared for assessment of radioactivity incorporated into protein as described in the text. The total radioactivity per dish (mean of duplicates) is plotted as a function of time after MNNG was added to the medium, and demonstrates the alteration of activity in cells exposed to MNNG, compared to controls.


3
2
1
MNNG
10μM
control
25μM
0
1
2
3
4
5
time (hours) at 37° after MNNG addition
[14C] amino acid incorporation (10-3 d. p. m. /dish)

Compound	R_f value
Thymidine	0.73 (0.66)
TMP	0.48 (0.44)
TDP	0.39 (0.30)
TTP	0.31 (0.24)

Table 15. Chromatography of acid soluble extracts of cells: R_f values.

The R_f values (mean of four runs) are shown for thymidine and its mono-, di-, and triphosphorylated derivatives obtained in the experiments described in the text. The values, with the same solvent system, obtained by Keir and Smellie (1959) are given in parentheses.

Figure 28. Incorporation of $[^3H]$ thymidine into phosphorylated derivatives of thymidine by cells, after exposure to MNNG.

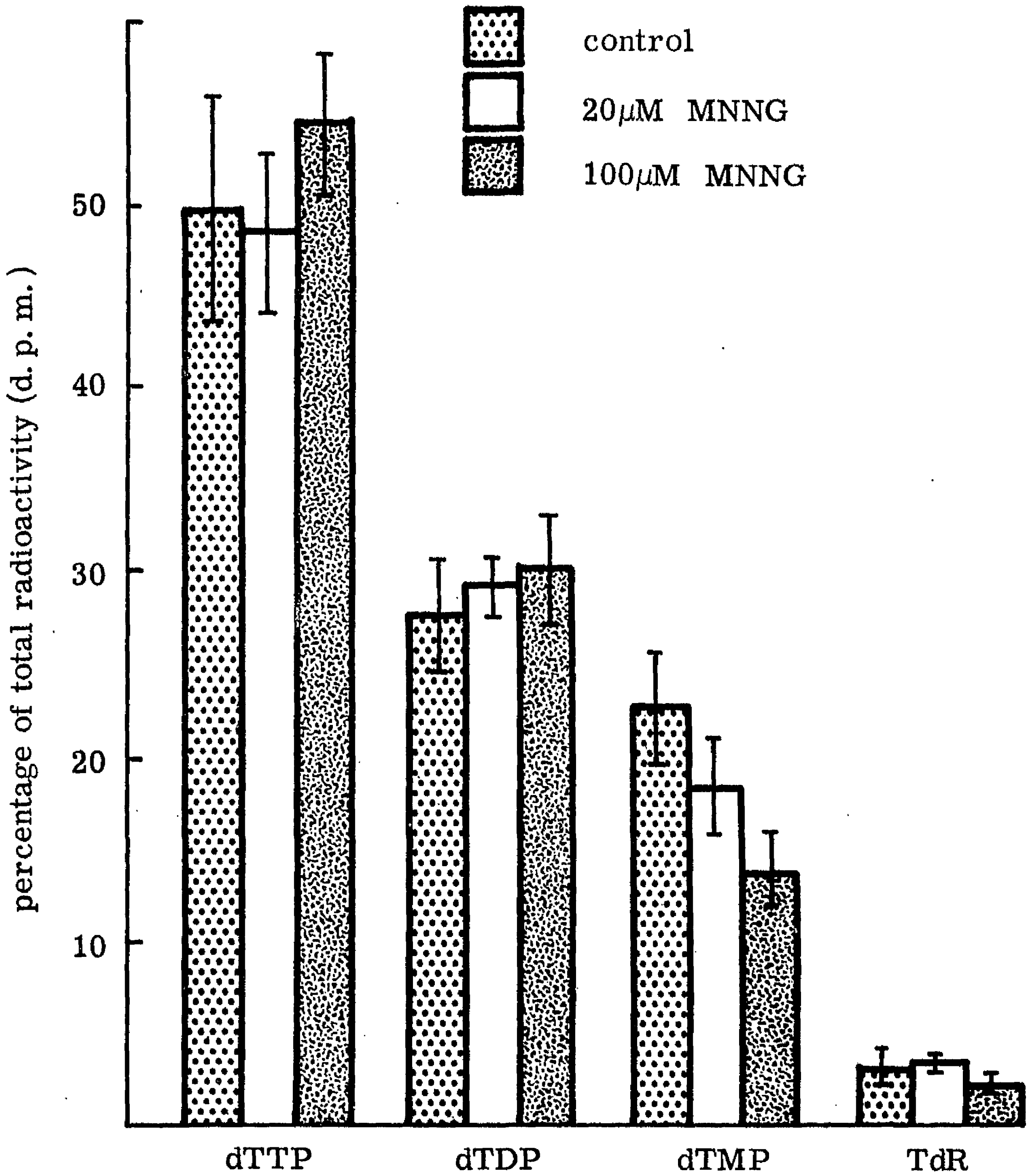
Aliquots of acid soluble extracts prepared from MNNG- and control-treated cells were chromatographed, and the separated constituents assessed for radioactivity, according to procedures outlined in the text. The distribution of radioactivity among thymidine and its mono-, di- and triphosphorylated derivatives is shown for each as the percentage of total radioactivity per run (four separate extracts for each treatment, mean of four runs $\pm$ SEM.)

The persistence of phosphorylation of thymidine in MNNG-treated cells to an extent equivalent with controls is evident. The total radioactivity extracted from the different cell populations was as follows (x 10^{-4} d.p.m./dish $\pm$ SEM)

Control .... 2.52 $\pm$ 0.75

20 μM MNNG .... 2.18 $\pm$ 0.41

100 μM MNNG .... 2.56 $\pm$ 0.25


control
20μM MNNG
100μM MNNG
percentage of total radioactivity (d. p. m.)
50
40
30
20
10
dTTP
dTDP
dTMP
TdR

3. <u>Experiments with cell extracts of Chinese hamster cells</u>.

a. DNA polymerase activity of cell extracts

A comparative examination was made of the activity of the cytoplasmic and nuclear extracts obtained according to III, B.5.b., and assayed according to III, B.5.e. The results are shown in Table 16, from which it is evident that the maximum activity was present in the cytoplasmic extract (CE), and the optimum priming activity was given by denatured DNA. In all subsequent experiments the activity of only the CE from the cells, with denatured DNA, was tested.

The effect of increasing the concentration of CE, or of the DNA primer, in the assay system was examined; the results are presented in Fig. 29. From this study, concentrations per assay of approximately 200 µg. protein, for CE, and 100 µg. DNA primer were employed in the assay system. At these concentrations the maximum rate of enzyme activity was obtained, whilst at higher (and lower) concentrations the rate of reaction was decreased. This observation is important since it indicates firstly, that the enzyme in the reaction was inhibited by high concentrations of the substrate (DNA), and secondly, that for the detailed study of enzyme inhibition by MNNG (see g. below), concentrations of less than 200 µg./assay of CE protein must be used. This latter precaution must be taken in order that enzyme inhibition is not masked by excess of enzyme.

b. Inhibition of DNA polymerase activity by MNNG

The effect on the activity of CE of addition, to the standard assay system, of MNNG at various concentrations is shown in Fig. 30a. The inhibition that resulted was delayed in its effect and did not appear to show a direct relationship to the concentration of MNNG, such as that already shown for the inhibition of $\left[^{3}H\right]$thymidine incorporation into DNA of the cells (III, C.3.a.iii.). However, it was found that prior incubation of CE with MNNG, before the addition of the other components of the assay system, allowed a more sensitive detection of the inhibitory effect. The effect of increasing the preincubation time of CE with MNNG prior to

Table 16. DNA polymerase activity of cell extracts with different DNA primers.

Aliquots of cell extract (100 μg protein/assay) were assayed for activity in the standard reaction mixture as described in text. The primer DNA was either native or thermally denatured DNA (100 μg/assay) and activity was based on the amount of $[^{32}P]$ dTMP, from $[^{32}P]$ dTTP, incorporated into an acid insoluble form after 2 hr. incubation at 37^{o}. Absolute quantities were calculated from the known specific activity of the radioactive precursor. DNAse was included in the reaction mixture at 100 μg/assay, where indicated.

Preparation	Primer	mμmoles/mg. prot./hr., incorp. $[^{32}P]$dTMP
Cytoplasmic extract	Denatured DNA	1.07
Cytoplasmic extract	Native DNA	0.34
Nuclear extract	Denatured DNA	0.78
Nuclear extract	Native DNA	0.22
Cytoplasmic extract	No primer	not above background
Cytoplasmic extract	Denatured DNA plus DNAse	not above background

Figure 29.

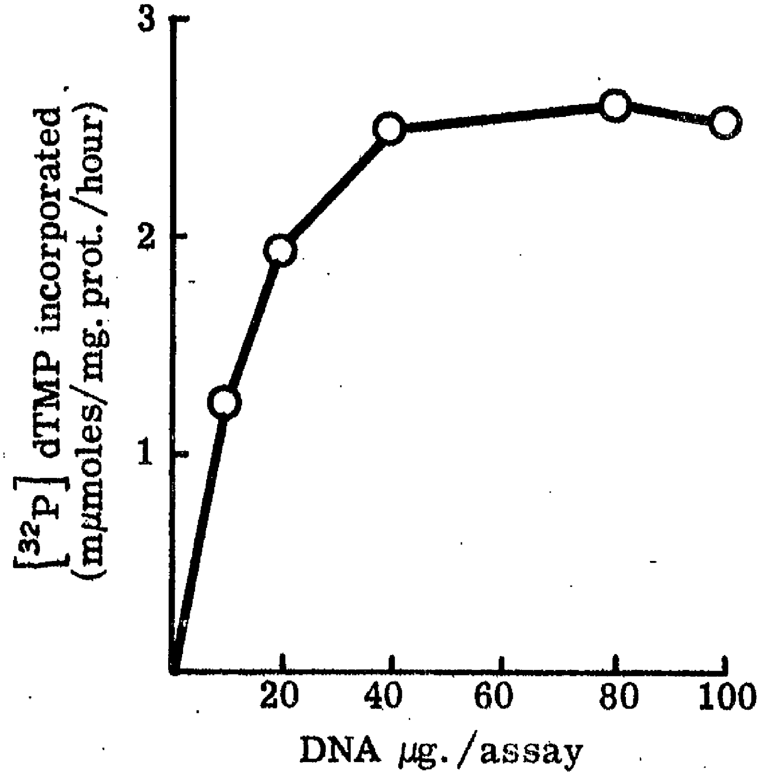
(a) DNA primer concentration and optimum DNA polymerase activity.

The composition of the standard reaction mixture described in the text was modified to include denatured DNA primer at different concentrations. Activity of cytoplasmic extract (170 μg protein/assay) was assayed according to standard procedures, and $[^{32}P]$ radioactivity incorporated was converted to absolute amounts from the known specific activity values. Activity, as mμmoles $[^{32}P]$ dTMP/mg.prot./hr. (mean of duplicate), is plotted as a function of DNA concentration (μg./assay).

(b) Cell extract concentration and optimum DNA polymerase activity.

The standard reaction mixture described in the text was used. Aliquots of cytoplasmic extract (CE) were added and the mixture incubated 90 min at 37°; incorporated radioactivity was assayed according to standard procedures and converted to absolute amounts from the known specific activity values. Activity, as mμmoles $[^{32}P]$ dTMP/mg.prot./hr. (mean of duplicates), is plotted as a function of CE concentration (μg. protein/assay).

(a)


3
2
1
[32P] dTMP incorporated
(mμmoles/mg. prot./hour)
20
40
60
80
100
DNA μg./assay

(b)

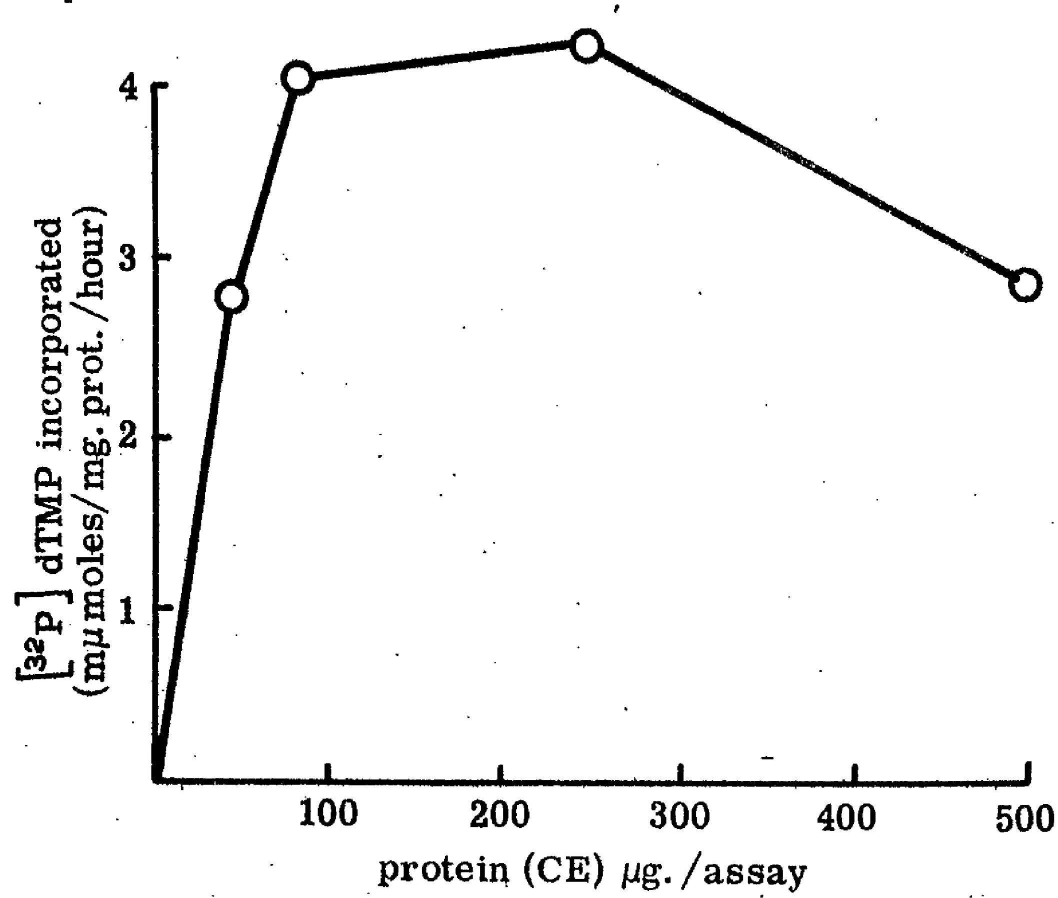

4
3
2
1
[32P] dTMP incorporated
(mμmoles/mg. prot./hour)
100
200
300
400
500
protein (CE) μg./assay

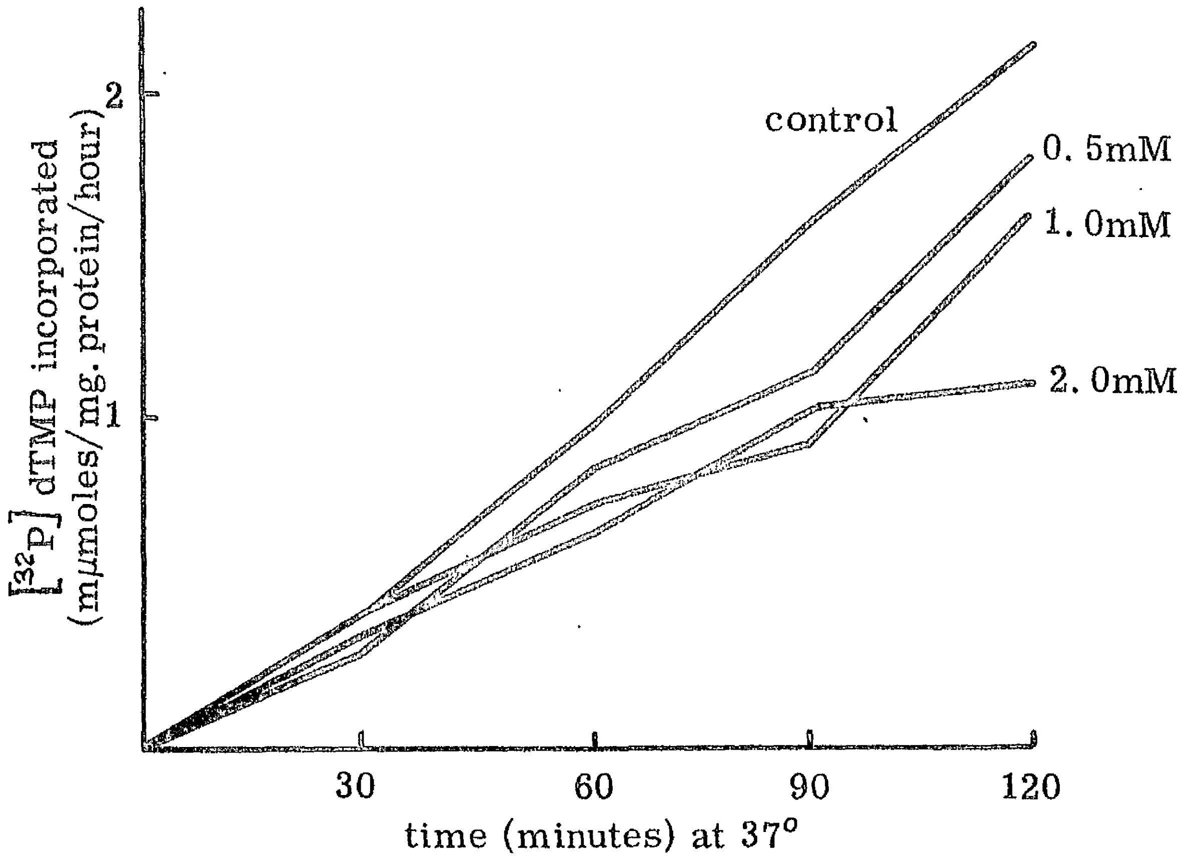
Figure 30. Effect of MNNG on DNA polymerase activity of cell extracts.

(a) DNA polymerase activity of cytoplasmic extracts assayed in the presence of MNNG.

Cytoplasmic extracts (170 µg.protein/assay) were assayed for activity according to standard procedures, in reaction mixtures composed as described in the text but which also contained MNNG at the initial concentration indicated on the graph. Samples were withdrawn at 30 min. intervals for assay of incorporated radioactivity, as described in the text, which was converted to absolute amounts from the known specific activity values. The increase in amount incorporated is plotted as a function of increase in incubation time. No clear relationship between inhibition of activity and initial concentration of MNNG is apparent.

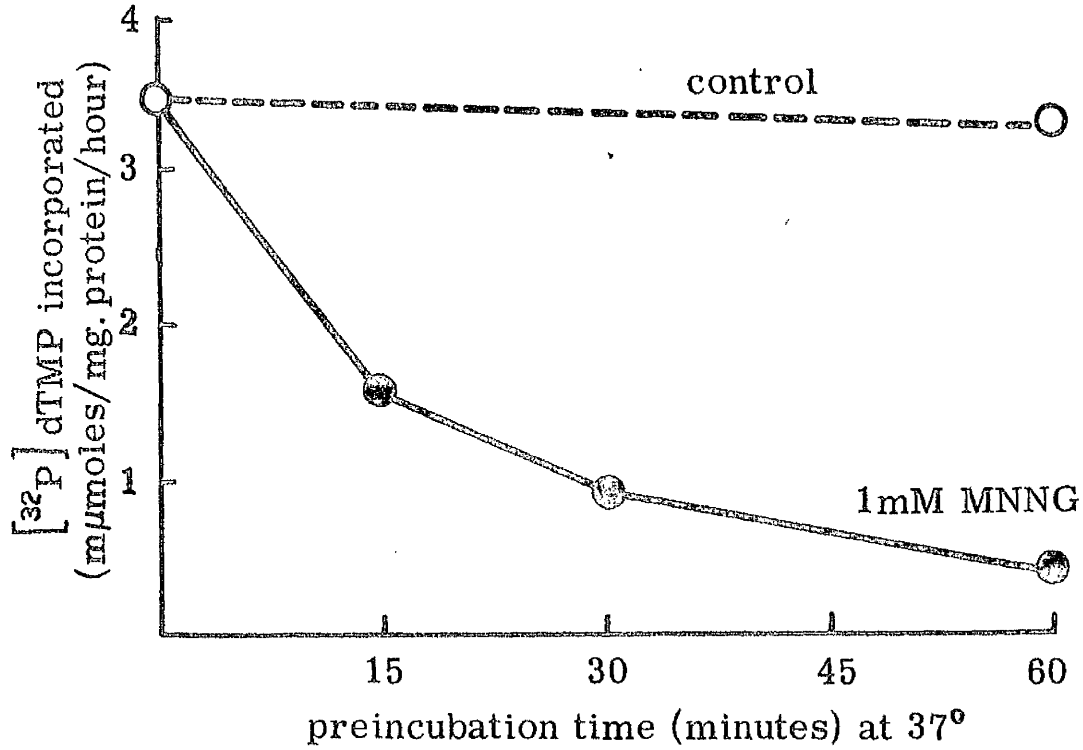
(b) Effect of preincubation with MNNG on DNA polymerase activity of cytoplasmic extract.

Aliquots of cytoplasmic extract (170 µg. protein/assay) were preincubated alone with 1 mM MNNG, for different periods at 37^{o}, before the other constituents of the reaction mixture were added. Incubation at 37^{o} was continued for 90 min. before samples were withdrawn for assessment of incorporated radioactivity according to standard procedures. Radioactivity was then converted to absolute amounts from the known specific activity values. The activity, as mµmoles $[^{32}P]$ dTMP/mg.prot./hr., is plotted according to duration of preincubation with MNNG. The significance of the decrease in activity and its relationship to length of preincubation with MNNG are discussed in the text.



[^{32}P] dTMP incorporated
(mμmoles/mg. protein/hour)
2
1
control
0. 5mM
1. 0mM
2. 0mM
30
60
90
120
time (minutes) at 37°

(a)



[^{32}P] dTMP incorporated
(mμmoles/mg. protein/hour)
4
3
2
1
control
1mM MNNG
15
30
45
60
preincubation time (minutes) at 37°

(b)

the assay of activity under standard conditions (III, B.5.e.) is shown in Fig.30b. These results provide an approximation of the rate of the inhibition process.

The effect of preincubation of CE aliquots for 60 min. with different concentrations of MNNG, before assay of activity under standard conditions, is shown in Fig. 31, and demonstrates a significantly greater dose-response than observed previously (Fig.30a).

In all subsequent experiments of the inhibition of DNA polymerase activity by MNNG a preincubation period of 60 min. was used.

c. The effect of guanidine hydrochloride on DNA polymerase activity

In view of the activity of MNNG as an inhibitor of DNA polymerase activity (b., above), the effect of the 'parent' compound, guanidine, on the reaction was also examined. Eggers and Tamm (1963) considered, as a possible explanation of the inhibition of virus multiplication by 0.1 mM guanidine, an interference by guanidine with the tertiary or quaternary structure of the virus-induced RNA polymerase. The concentration reported by Groves, Hipp and McMeekin (1951) to cause instantaneous denaturation of protein was 5 M guanidine.

The DNA polymerase activity of CE after it was preincubated for 60 min. with 1mM guanidine HCl is shown in Fig. 32. The inhibition was of the order of 25%, as compared to the inhibition of around 60% by MNNG at the same molarity when both were assayed in the presence of 2-mercaptoethanol (see f. below).

The inhibition of nuclear extract was not examined.

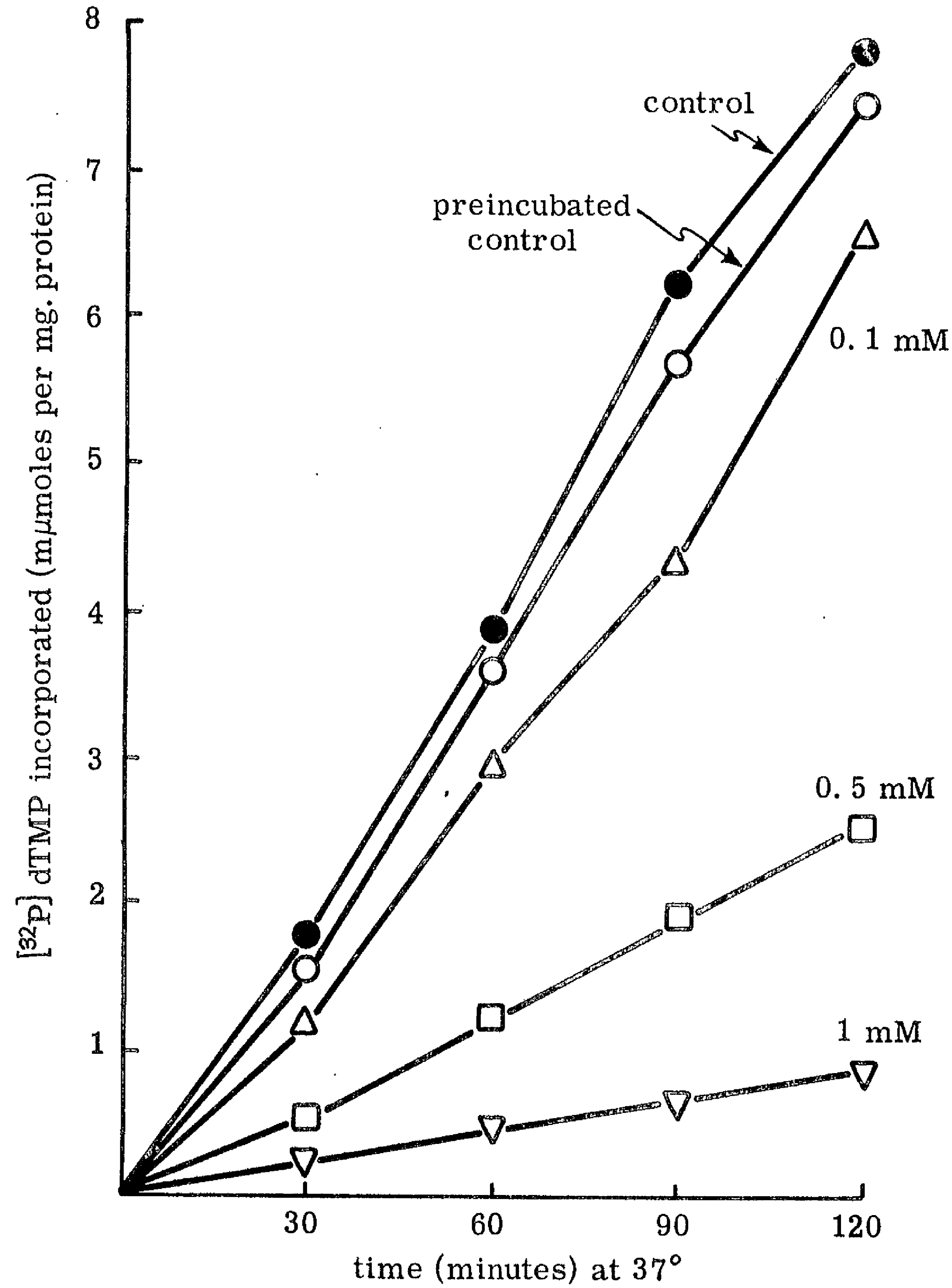
d. DNA polymerase activity with primer DNA that has been pretreated with MNNG

Although DNA polymerase activity was decreased by preincubation of enzyme (CE) with MNNG (b., above) the significance of MNNG interaction with the DNA 'primer' component of the assay system was also examined. Calf thymus (Sigma Chemical Company) was treated, in bulk, as described (III, B.6.), and then used as primer, both native and denatured, in the

Figure 31. MNNG inhibition of DNA polymerase activity of cell extracts.

MNNG or control buffer was added to aliquots of cytoplasmic extract from untreated cells and the mixture incubated for 60 min. at 37° before the other constituents of the standard reaction mixture were added and DNA polymerase activity was assayed according to procedures outlined in the text. Samples were taken at 30 min intervals from the reaction mixture (200 µg. protein/assay) for assessment of radioactivity incorporated, as described in the text, which was converted to absolute amounts from known specific activity values. Activity is plotted as incorporated precursor, in mµmoles/mg.protein (mean of duplicates) against the duration of incubation.

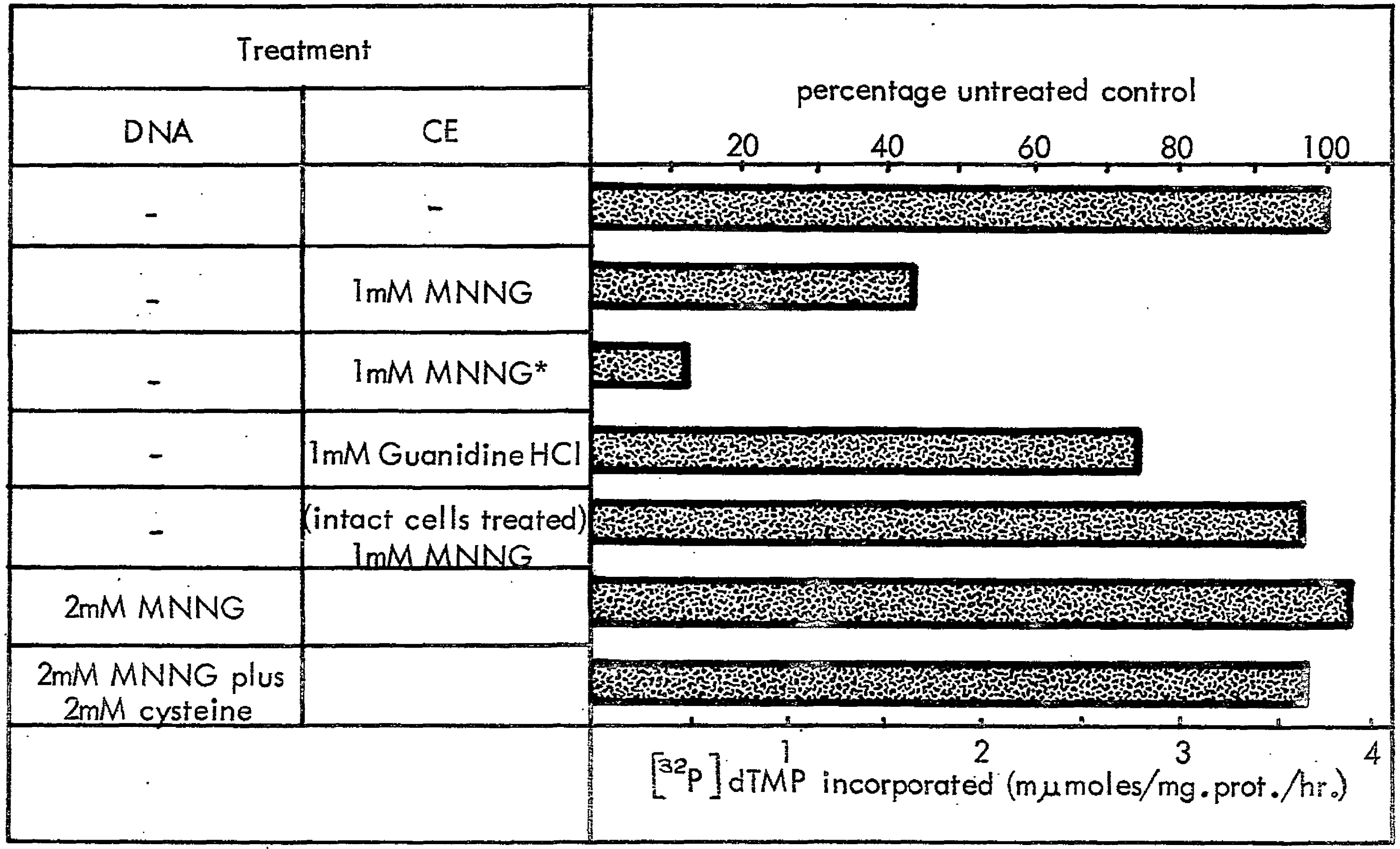
A dose dependent inhibition of activity by MNNG is evident; preincubation did not significantly alter control activity.



8
7
6
5
4
3
2
1
[^{32}P] dTMP incorporated (mμmoles per mg. protein)
control
preincubated control
0. 1 mM
0. 5 mM
1 mM
30
60
90
120
time (minutes) at 37°

Figure 32. Comparison of pretreatments of DNA primer or cell extract on DNA polymerase activity.

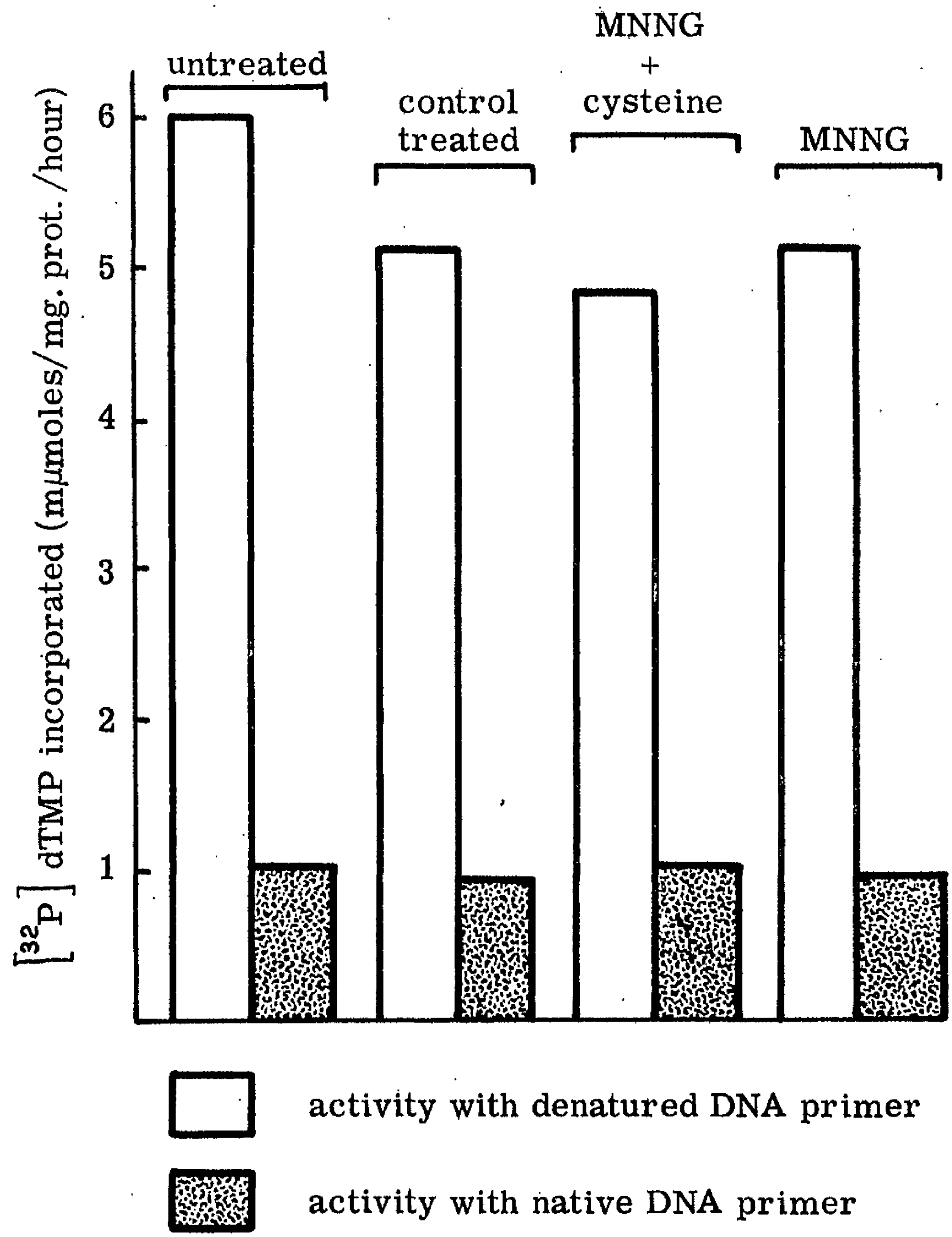
DNA primer or cytoplasmic extract (CE) was preincubated at 37° for 60 min., with additions as indicated, and then assayed for activity according to standard procedures described in the text. Activity is shown (mean of duplicates) both as the absolute amount incorporated per hour (250 μg. protein/assay) and as percentage of untreated control (preincubated). The significance of the different activities is discussed in the text.



* CE and assay mixture without 2-mercaptoethanol

Figure 33. DNA pretreated with MNNG as primer of DNA polymerase activity.

DNA was treated in bulk with MNNG, as described in the text, and used in the standard reaction mixture, in the native or denatured state (100 μg/assay), as primer of DNA polymerase activity with cytoplasmic extract of cells (220 μg protein/assay). The pretreatment is indicated above the appropriate histogram. Absence of significant differences in activity (absolute amount of precursor incorporated per hr., mean of duplicates) after treatment is evident.



[32P] dTMP incorporated (mμmoles/mg. prot./hour)
6
5
4
3
2
1
untreated
control treated
MNNG + cysteine
MNNG
activity with denatured DNA primer
activity with native DNA primer

standard assay system. The results are shown in Fig. 33.

The persistence of DNA polymerase activity with MNNG-treated DNA as primer was demonstrated. The significance of the 5% decrease in activity of CE with denatured primer that had been treated with MNNG in the presence of 2 mM cysteine was not understood, but may have been related to the increased extent of alkylation of DNA (approx. one in 10^4 nucleotides) under these conditions (McCalla, 1968b). Alkylated guanine (McCalla, 1968a; Craddock, 1968) and also adenine (Lawley, 1968) have been isolated from DNA treated with MNNG in vitro. The results of the present experiments did not suggest, however, that the alkylation of DNA primer by MNNG played a significant part in the inhibition of the DNA polymerase reaction in vitro by MNNG alone.

e. DNA polymerase activity of extracts from MNNG treated cells

Cells in bulk cultivation (III, B.5.a.) were exposed to MNNG at 60 µM for one hour before they were harvested and treated according to standard procedures for enzyme extraction (III, B.5.b.). This concentration of MNNG would be expected to cause almost 70% inhibition of $\left[^{3}H\right]$thymidine incorporation into DNA (see Fig. 25). Nevertheless, the activity of cytoplasmic extract from treated cells was equivalent to that of untreated cells (Fig. 32). Activity of CE with native primer, or of NE with native or denatured primer, was not examined.

f. The effect of various compounds on the extent of inhibition of DNA polymerase activity caused by MNNG

The ability of compounds to alter the inhibitory effects of MNNG was tested by observing the effect of their inclusion, along with MNNG, during the preincubation of the extract. Unless stated otherwise the aliquot of MNNG was added last.

Lysine (B.D.H.), a diamino acid, and histidine (B.D.H.), which carries a nucleophilic imidazole ring, each at 2 mM, and catalase (Sigma Chemical Co.) at 200 enzyme units/ml. were without effect on controls; there was also no alteration to the inhibition of DNA polymerase activity by MNNG. These results are shown in Fig. 34.

The sulphydryl compound, 2-mercaptoethanol, has been recommended (Keir and Shepherd, 1965) as a constituent of the buffers used to extract polymerase enzyme from animal cells, and to be incorporated in the polymerase assay system, at a concentration of 5 mM (in each). It was found, however, that the inhibition by MNNG was greater when 2-mercaptoethanol was omitted from both extraction buffer and the assay system. From the results of a comparative experiment, given in Fig. 32, it was evident that the inhibition by 1 mM MNNG increased from 60% to 85% in the absence of extrinsic thiol groups. 2-Mercaptoethanol was, therefore, excluded from the buffers used for enzyme extraction and the assay systems in subsequent experiments of MNNG dose-response (b., above) and enzyme inhibition kinetics (g., below). This modification had the disadvantage of allowing deterioration of enzyme activity, with substantial loss of activity one week after extraction, even with storage at 0^{o}. All enzyme preparations so obtained were thus used within the week of extraction.

The tripeptide, glutathione (GSH), was also tested for the ability to decrease the inhibition by MNNG of DNA polymerase activity in cell extracts prepared in the absence of added thiol groups. This compound gave the treatest 'protection' against inhibition by MNNG; at 2 mM in the preincubation mixture containing 1 mM MNNG the inhibition, on subsequent assay of DNA polymerase activity, was reduced from 85% to 25% (Fig. 34). The decrease in inhibition by MNNG resulting from the presence of different concentrations of glutathione in the preincubation mixture is shown in Fig. 35. In another experiment, to examine for reversibility of MNNG inhibition, glutathione was added only at the end of preincubation, that is, immediately before the addition of the other components (nucleotides etc.) of the assay system. No significant reversal was obtained (see Fig. 35).

Also shown in Fig. 35 is the virtually complete loss of activity with the inclusion of 5 mM glutathione, with or without MNNG, in the preincubation mixture. This effect was most probably due to the precipitate that formed in the reaction solution during incubation. A similar

Figure 34. Effect of various compounds on the extent of inhibition of DNA polymerase activity by MNNG.

Aliquots of cytoplasmic extract from untreated hamster fibroblasts were preincubated at 37^{o}, with additions as indicated, and then assayed (200 µg. protein/assay) for DNA polymerase activity, according to standard procedures outlined in text. The amount of radioactivity incorporated after 90 min. incubation at 37^{o} was converted to absolute amounts from the known specific activity values. Activity is shown both as absolute amount incorporated and percentage of untreated, preincubated control (mean of duplicates).

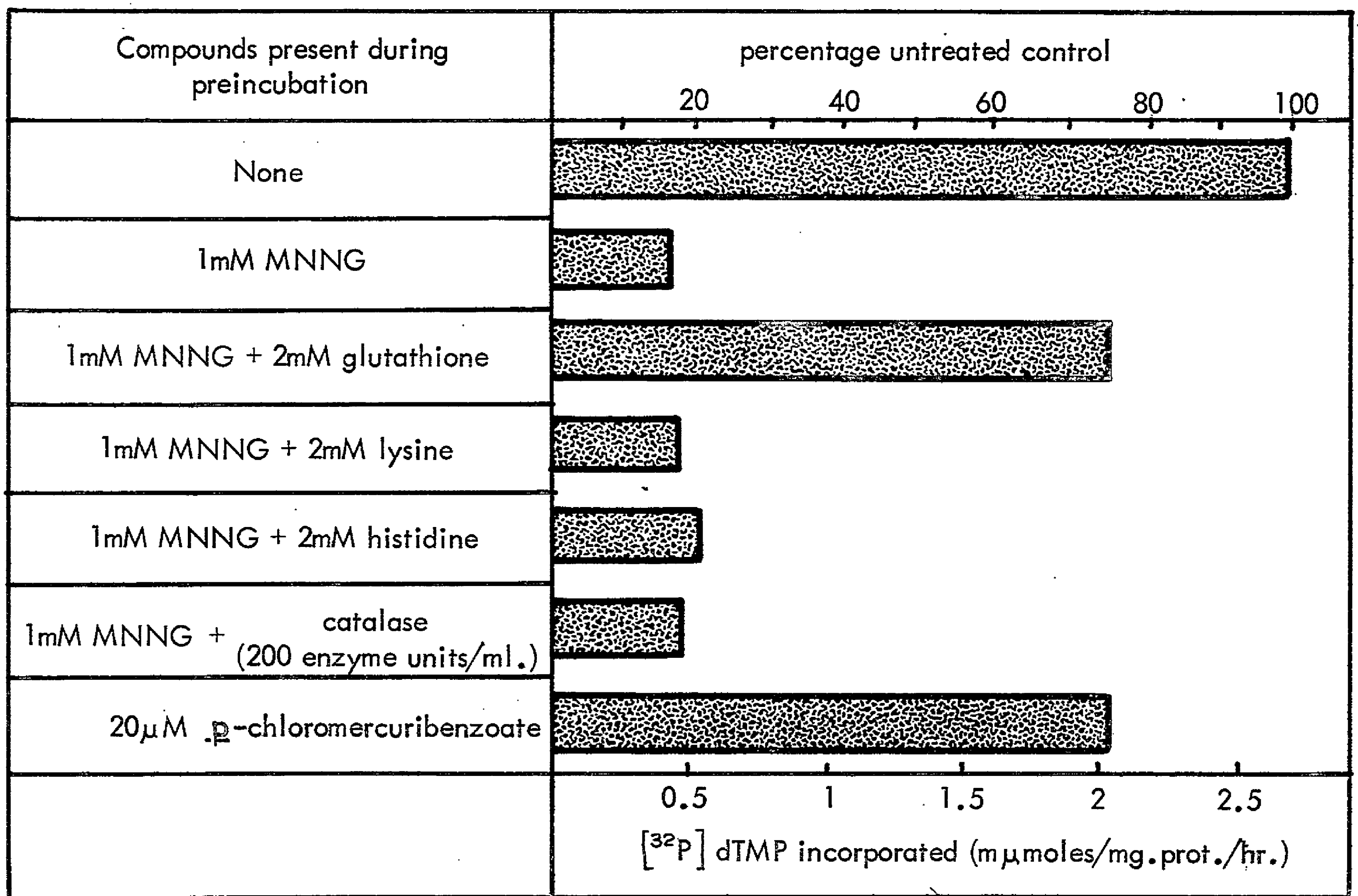
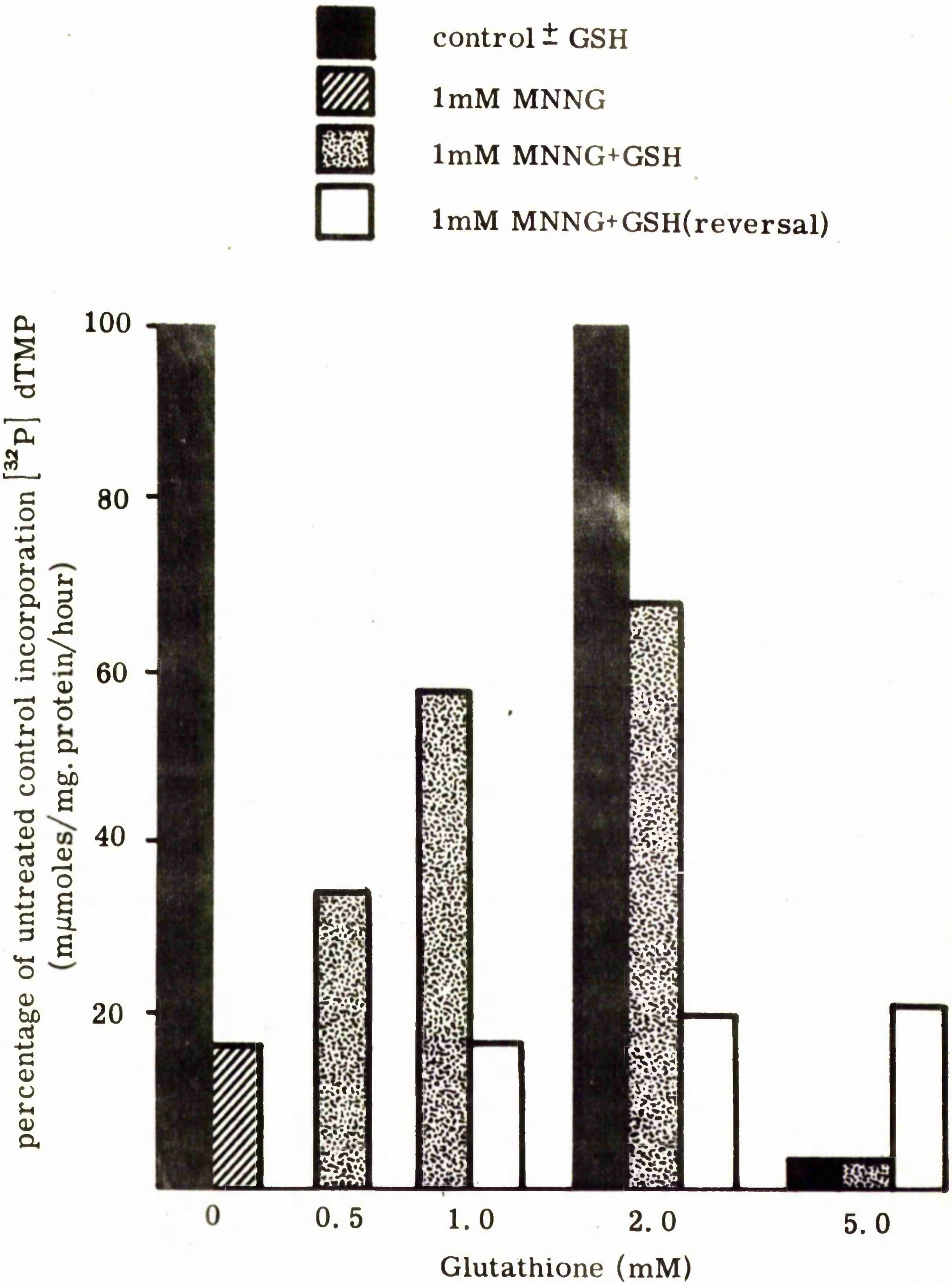

Compounds present during preincubation
percentage untreated control
20
40
60
80
100
None
1mM MNNG
1mM MNNG + 2mM glutathione
1mM MNNG + 2mM lysine
1mM MNNG + 2mM histidine
1mM MNNG + catalase (200 enzyme units/ml.)
20µM p-chloromercuribenzoate
0.5
1
1.5
2
2.5
[32P] dTMP incorporated (mµmoles/mg.prot./hr.)

Figure 35. Effect of glutathione on DNA polymerase activity and its inhibition by MNNG.

Aliquots of cytoplasmic extract from untreated cells were preincubated alone, with additions as indicated, for 60 min. before the other constituents of the standard reaction mixture were included and activity assayed according to procedures outlined in the text. To test for ability of glutathione (GSH) to reverse the inhibition by MNNG, GSH was added to samples with MNNG only at the end of preincubation, i.e., immediately before the other constituents were included.

Radioactivity per sample, at 90 min. incubation, was converted to absolute amounts of substrate incorporated (200 µg. protein/assay). Activity is expressed (mean of duplicates) as percentage of control without additions. The significance of results is discussed in the text.


control ± GSH
1mM MNNG
1mM MNNG+GSH
1mM MNNG+GSH(reversal)
percentage of untreated control incorporation [^{32}P] dTMP
(mμmoles/mg. protein/hour)
100
80
60
40
20
0
0.5
1.0
2.0
5.0
Glutathione (mM)

precipitate, believed to be protein, was noted by Keir and Shepherd (1965) at high molarities of glutathione, with Landschutz ascites tumour cell extracts.

Also tested for their ability to alter the inhibition of DNA polymerase activity by MNNG were the enzyme substrates, primer DNA or deoxyribonucleoside-5'-triphosphates. The results of a typical experiment are shown in Fig. 36, from which it is evident that MNNG inhibition is reduced (from 85% to 50%) in the samples that also contained the standard amount of DNA primer in the preincubation mixture. Caution must be exercised when assessing this reduction, however, as DNA polymerase activity of untreated aliquots preincubated with primer DNA, or primer DNA and deoxyribonucleoside triphosphates (dATP, dCTP, dGTP and dTTP), was increased approximately 25% and 50%, respectively, above controls without additions (Fig. 36).

The addition, to the preincubation solution of CE, of only the four deoxyribonucleoside triphosphates (see above) together, had no effect on the inhibition of DNA polymerase activity by MNNG. Moreover, the activity of control CE preincubated with the triphosphates was inhibited to around 50% of control without additions (see Fig. 36). Repetition of the experiment with supplements of fresh deoxyribonucleoside triphosphates at the end of preincubation gave results with a similar inhibition of activity. This latter finding was taken as evidence that the observed decrease in activity was due to factors other than dephosphorylation of deoxyribonucleoside triphosphate during preincubation. The phenomenon was not examined further.

g. The kinetics of DNA polymerase activity and its inhibition by MNNG.

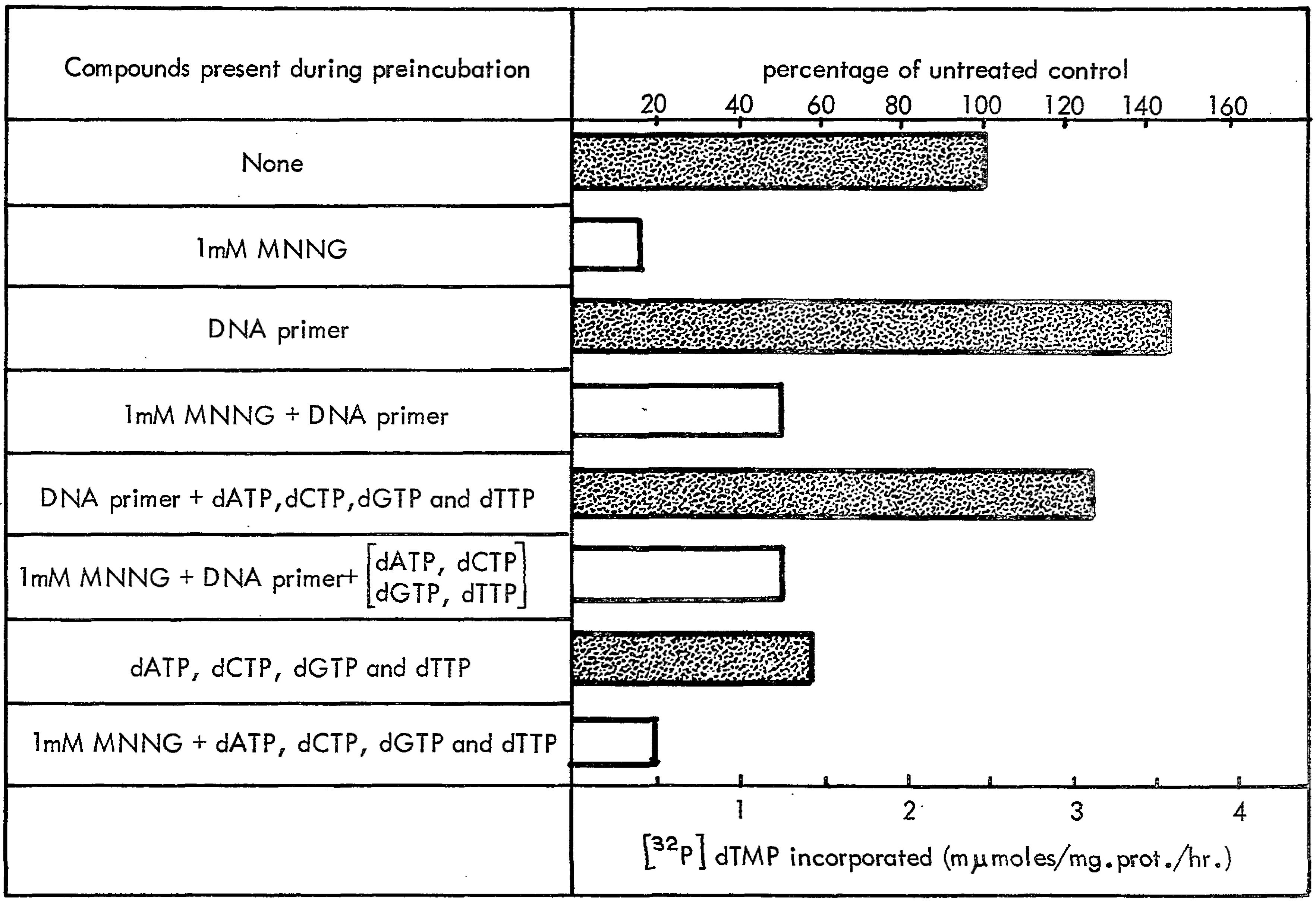
DNA polymerase activity of CE was assayed according to standard procedures (III, B.5.e.) with the following modifications.

(i) In one type of analysis, CE aliquots were assayed for activity with different concentrations of substrate (either DNA primer or deoxyribonucleoside triphosphates). The results of typical

Figure 36. <u>Effect of preincubation with substrates on DNA polymerase activity of cell extracts, and its inhibition by MNNG.</u>

Aliquots of cytoplasmic extract of untreated cells were preincubated 60 min. at 37^{o}, with additions as indicated, before the other constituents of the standard reaction mixture were included and the activity assayed as outlined in the text. Radioactivity per sample, at 90 min. incubation, was converted to absolute amounts of substrate incorporation (200 µg. protein/assay), and activity is shown as such, as well as the percentage of untreated control (mean of duplicates).

Decreased inhibition by MNNG is evident in the samples which contain also DNA primer. Note, also the increase in activity of controls treated similarly.



Compounds present during preincubation
percentage of untreated control
20
40
60
80
100
120
140
160
None
1mM MNNG
DNA primer
1mM MNNG + DNA primer
DNA primer + dATP, dCTP, dGTP and dTTP
1mM MNNG + DNA primer + [dATP, dCTP dGTP, dTTP]
dATP, dCTP, dGTP and dTTP
1mM MNNG + dATP, dCTP, dGTP and dTTP
1
2
3
4
[32P] dTMP incorporated (mμmoles/mg.prot./hr.)

experiments are presented graphically, in Fig. 37, as the double reciprocal plot according to Lineweaver and Burk (1934). The alteration to results caused by preincubation of CE with 1 mM MNNG, for 60 min., to be subsequently assayed with various concentrations of DNA primer, is represented graphically in the same manner in Fig. 38, for comparison against results with preincubated control CE.

(ii) In another type of analysis, CE aliquots were preincubated for 60 min. with different concentrations of MNNG, each to be assayed at both of two concentrations of DNA primer. The results are presented graphically in Fig. 39.

The interpretation of these results (i and ii, above) are discussed later (III, D.3.b.iii.).

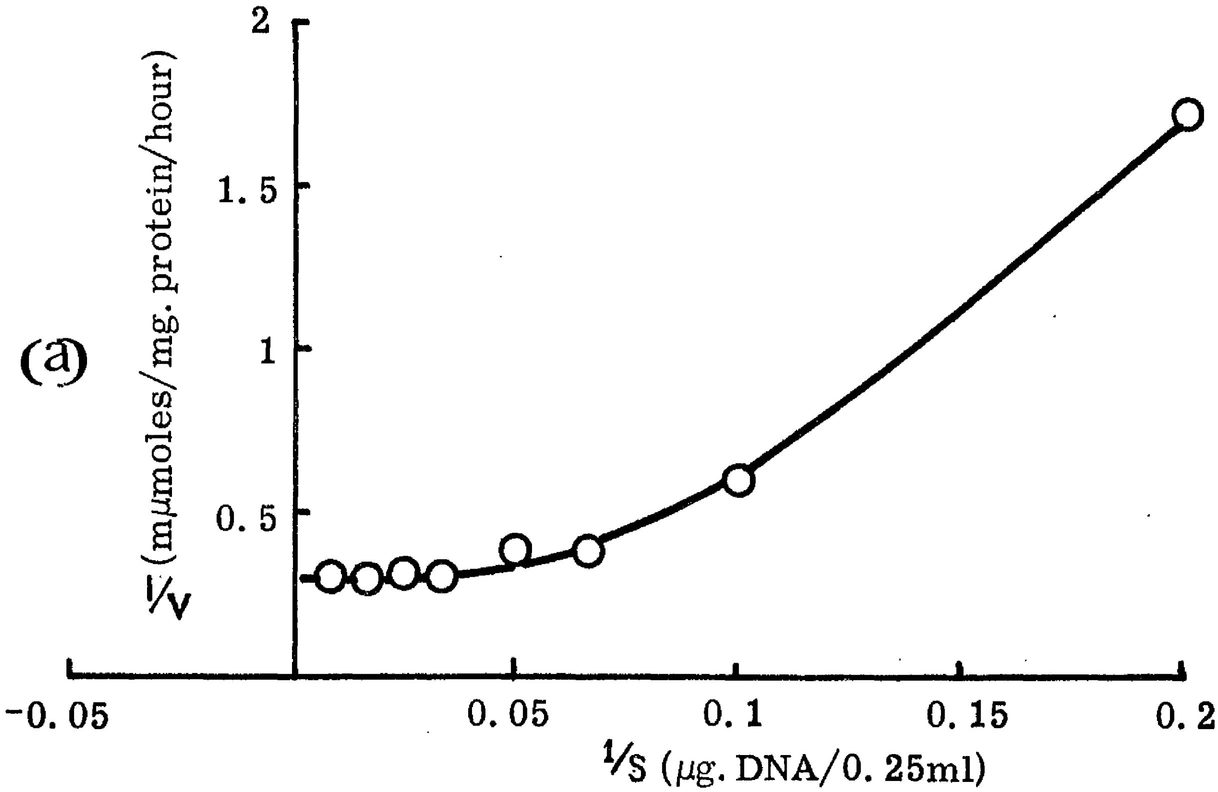
Figure 37. DNA polymerase reaction kinetics: double-reciprocal plots.

Aliquots of cytoplasmic extract from untreated cells were assayed for DNA polymerase activity according to standard procedures outlined in text at different substrate concentrations. Radioactivity was converted to absolute amounts and is expressed as reaction velocity (V) in terms mμmoles of $[^{32}P]$ dTMP incorporated/mg. protein/hr. (mean of duplicates; samples at 90 min. incubation; 100 μg. protein/assay).

(a) Substrate concentration (S) is in terms of μg. DNA/assay. dATP, dCTP, dGTP and $[^{32}P]$ dTTP present at 50 mμmoles each/assay.

(b) Substrate concentration (S) is in terms of mμmoles triphosphate/assay. Denatured DNA primer present at 100 μg./assay.

The results are interpreted in the text.


(a)
2
1.5
1
0.5
1/V (mμmoles/mg. protein/hour)
-0.05
0.05
0.1
0.15
0.2
1/S (μg. DNA/0.25ml)

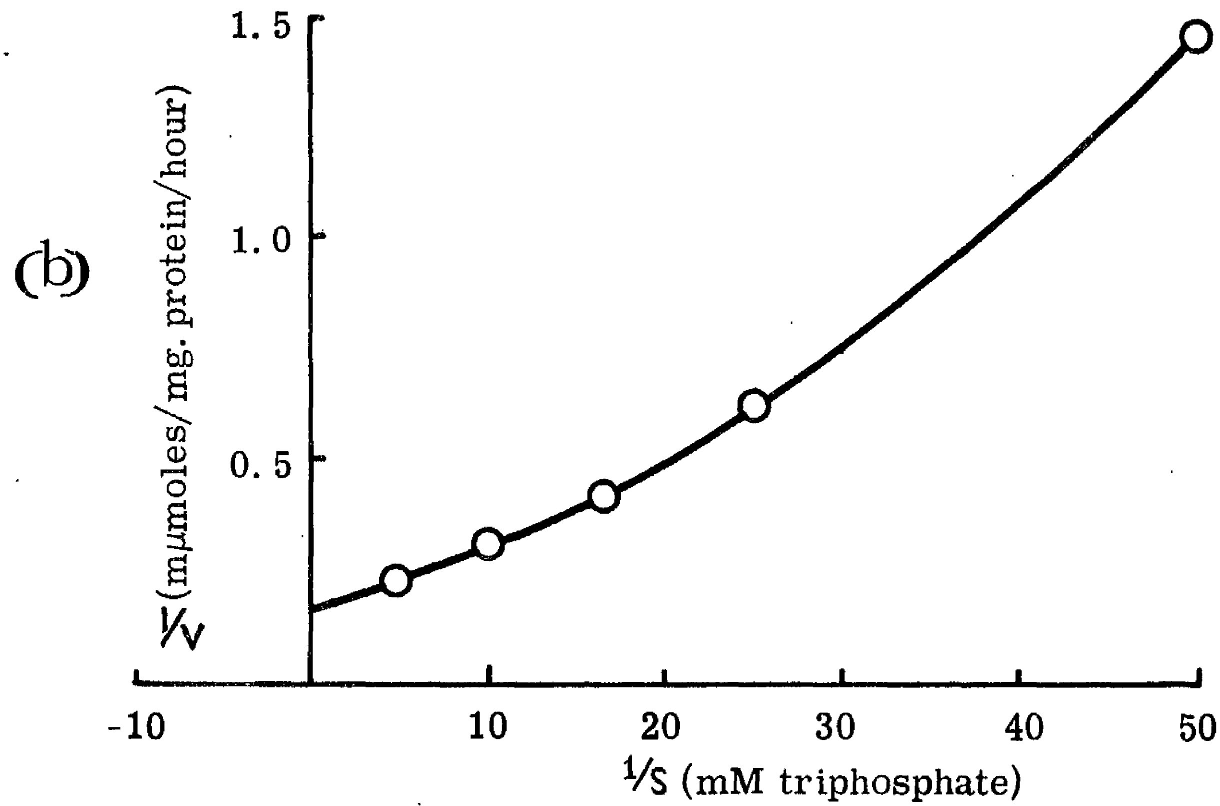
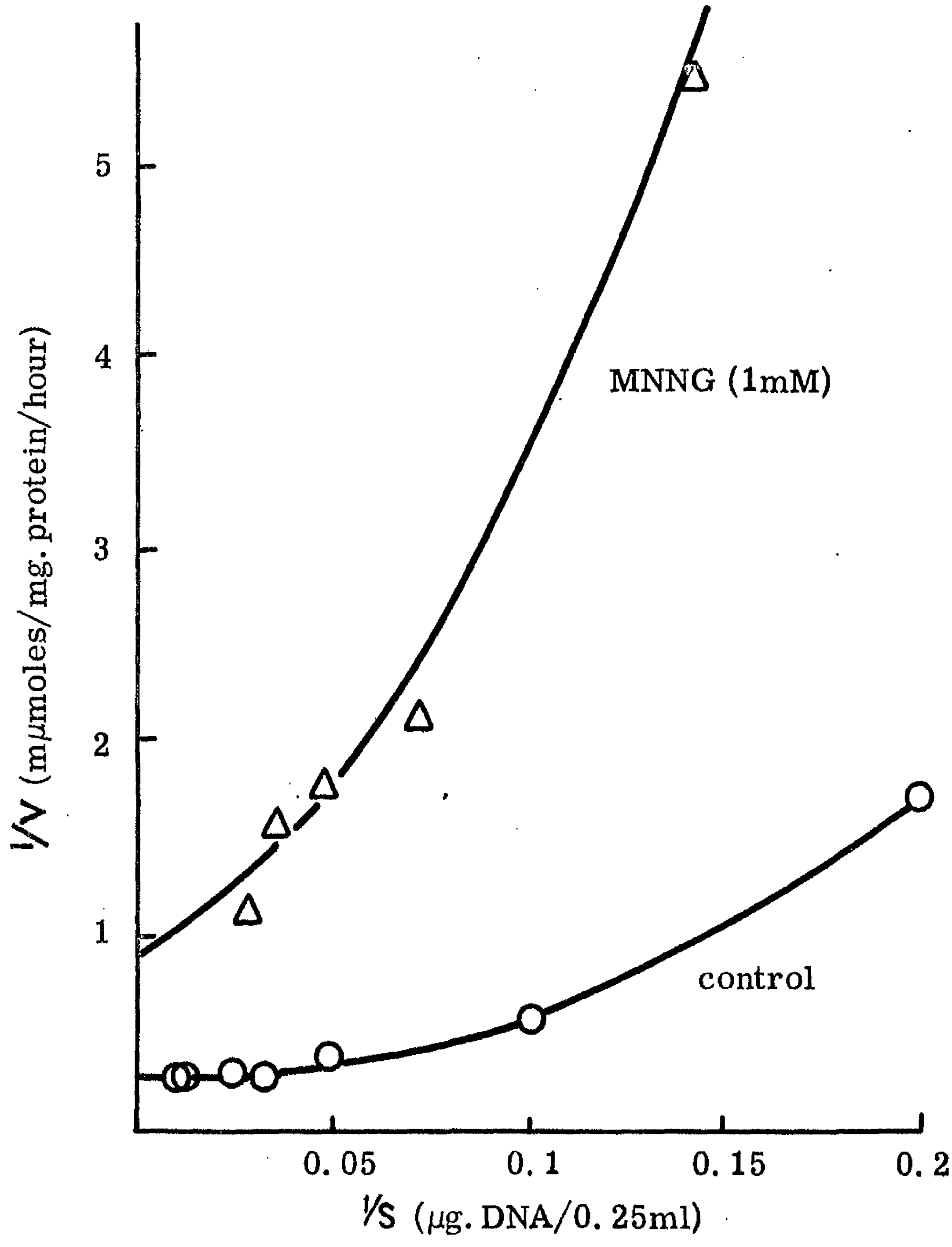

(b)
1.5
1.0
0.5
1/V (mμmoles/mg. protein/hour)
-10
10
20
30
40
50
1/S (mM triphosphate)

Figure 38. Inhibition of DNA polymerase activity by MNNG: reaction kinetics.

Aliquots of cytoplasmic extract from untreated cells were preincubated 60 min. with and without MNNG (1 mM, initial) and then assayed according to standard procedures, at different DNA (denatured) substrate concentrations (100 μg. protein/assay). Activity is expressed as reaction velocity (V) in terms of mμmoles $[^{32}P]$ dTMP incorporated/mg. protein/hr. (means of duplicates; samples at 90 min. incubation.)

The results are illustrated as the double-reciprocal plots, which are non-linear. Their significance is discussed in the text.

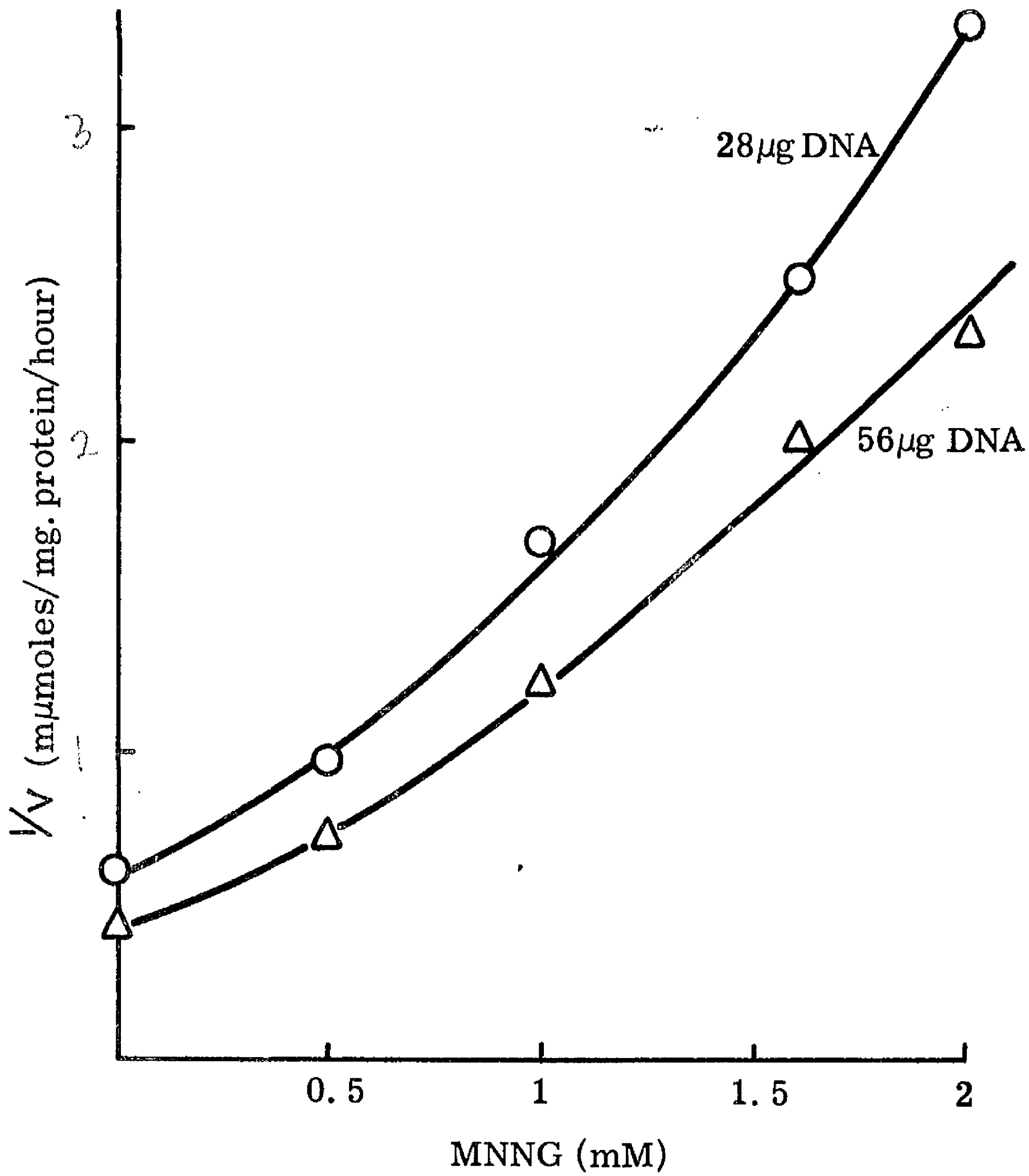
The substrate concentration (S) is in terms of μg. DNA/ assay.



1/V (mμmoles/mg. protein/hour)
5
4
3
2
1
MNNG (1mM)
control
0.05
0.1
0.15
0.2
1/S (μg. DNA/0.25ml)

Figure 39. Inhibition of DNA polymerase activity by MNNG: reaction kinetics with inhibitor concentration varied.

The experiment was as described for Figure 38, only aliquots were preincubated 60 min. with different concentrations of MNNG <u>and</u> DNA primer(at either of two concentrations)also included. Assayed according to standard procedures, activity is expressed as inhibited reaction velocity (V) in terms of mμmoles $[^{32}P]$ dTMP incorporated/ mg. protein/hr. (mean of duplicates; samples at 90 min. incubation, 100 μg. protein/assay.) The reciprocal of V, plotted against inhibitor concentration (initial), is non-linear for each DNA concentration.


28µg DNA
56µg DNA
1/V (mµmoles/mg. protein/hour)
3
2
1
0.5
1
1.5
2
MNNG (mM)

D. Discussion

The results of the present study of the effect on mammalian cell systems of exposure to MNNG can be conveniently classified into groups, based on the principal effect observed, as follows:-

1. Growth inhibition and cytotoxicity
2. Inhibition of macromolecular synthesis (DNA, RNA, protein)
3. Enzyme inhibition, *in vitro*.

1. Growth inhibition and cytotoxicity.

a. Growth inhibition and the cell cycle

The assessment of cell number after treatment with MNNG at a concentration of 5 μM, or more, reveals a rapid inhibition of cell multiplication (Figs. 17 and 18). Recovery, as judged by cell division, is evident within approximately 5 hr. of treatment with 20 μM MNNG, or less; to a limited extent division also occurs in populations treated with 25 μM MNNG at high cell number per dish (Fig. 18). A population of cells in exponential growth is composed of cells at all stages of the cell cycle, namely, G_1, S, G_2 and mitosis. From kinetic studies on cell populations, Lajtha, Oliver and Gurney (1962) propose a fifth phase, G_0, or "no cell cycle", from which cells may be stimulated to re-enter the cycle of division. As a possible explanation of this anomalous response, at 25 μM MNNG, of cells exposed at high population density it is proposed that the proportion of cells in G_0 becomes larger as the cell number per dish increases. If the cells of this phase are insensitive to MNNG, or at any rate less so, but are stimulated to re-enter the cell division cycle, a small increase in cell number would be expected. It is interesting that a similar alteration in response is observed in the studies of cytotoxicity, as judged by colony forming ability (Fig.19). However, measurement of cell number alone is insufficient to incriminate particular phase of the cell cycle as showing sensitivity to MNNG.

It is possible to deduce sensitivity of cells in G_2 phase to MNNG from the immediate decrease in mitotic index observed in a population of cells in exponential phase of growth, treated with 10 μM MNNG (Fig. 22b). Thus, in the absence of evidence that cells were lysed during their passage through the phase of mitosis, the decrease in the mitotic index must be due to failure of cells to pass through G_2 at the normal rate. Assuming values for the duration of G_2 ($2\frac{1}{2}$ hours) reported by Hsu, Dewey and Humphrey (1962) for Chinese hamster fibroblasts, the persistence of mitotic activity at three hours after treatment indicates either an incomplete arrest, or interference with the completion of mitosis. As the mitotic index in MNNG treated populations of cells did not decrease to zero, the sensitive event, related to time, in G_2 phase could not be determined. From the duration of the interval between X-irradiation (1000 rad) and decrease to zero mitotic index in cultured mouse cells, Watanabe and Okada (1966) calculated the sensitive period in G_2 phase to be approximately 0.7 hr. before mitosis. This result agrees with that of Puck and Steffen (1963) who report the sensitivity of HeLa cells to X-irradiation (9 rad) to be in G_2 phase at the point 0.6 hr. preceding the entry of cells into mitosis. Wheeler (1967) considers that the significance of the sensitivity of cells in G_2 phase to treatment with nitrogen mustard (~~allyl isothiocyanate~~, HN2) may be related to interference with protein synthesis required by cells to enter into mitosis.

Further information on the kinetics of growth inhibition by MNNG might be obtained from experiments with greater concentrations of MNNG (e.g. 25 μM). However, analysis of cell population kinetics, to determine the sensitivity of the different phases of the cell cycle was not continued. Studies of incorporation of radioactive precursor into DNA showed that cells in S phase, the phase of DNA synthesis, were affected by exposure to MNNG. The cells were also delayed in their passage through G_1 phase into S phase of the next cell cycle, as judged by the failure (see Fig. 23) of cells treated with 10 μM MNNG to maintain a normal, recovered rate of incorporation of $[^3H]$ thymidine beyond 7-8 hr. after treatment (assuming $G_2 + M + G_1 = 6$ hr., Hsu _et al_., 1962). These indications of the sensitivity of events in S, G_2, and in G_1 of the next cycle, suggest that there are many "targets" within the cell for reaction with MNNG, or the active agent derived from it. This is supported by studies of the cytotoxic effect of MNNG.

b. Cytotoxicity

The dose-response curves of survival in cells treated with MNNG, as judged by subsequent colony formation, show an initial shoulder followed by an exponential effect. A similar curve in the graph of survival in cells resistant to treatment with the alkylating agent, sulphur mustard (bis-(2-chlorethyl)-sulphide, HS2), is interpreted by Crathorn and Roberts (1966) to indicate the existence in these cells of a repair mechanism, which is capable of replacing DNA segments that contain alkylated nucleosides. Since MNNG has been shown (Craddock, 1968; McCalla, 1968a; Lawley, 1968) to alkylate DNA in vitro it is possible that a similar mechanism capable of replacing nucleosides of DNA alkylated by MNNG in vivo, accounts for the observed result.

However, as opposed to the bifunctional activity of HS2 that causes a proportion of cross-links between DNA strands, MNNG is expected to be monofunctional. Such monofunctional activity may be expected to be less toxic than bifunctional activity, and an alternative explanation to the above may be given, such as the existence of many targets within the cell. The target theory of cellular inactivation by irradiation gives, as the interpretation of survival curves that show an initial shoulder preceding the exponential decrease, the existence of two or more targets which must be "hit" in order for cellular inactivation to occur (Powers, 1962). The implications of this concept of "target" and "hits" has importance since the extrapolation of the straight line portion of the graph may be used to estimate the number of targets involved. For complex biological systems, such as mammalian cells, the precise meaning of such curves is not clear (Little, 1968), and therefore, the application of this theory to MNNG toxicity in this case is only speculative.

The cytotoxicity of other metabolic inhibitors, expressed as the dose required to reduce survival to 50% of control values, or ID_{50}, is shown in Table 17 for comparison against results with MNNG. The potent cytotoxic effect of the bifunctional alkylating agent, HS2, for mammalian cells is related to its ability to cross link the individual strands of DNA (Crathorn and Roberts, 1966; Lawley, 1966). However, the monofunctional alkylating agent, methyl methanesulphonate (MMS) is less toxic than MNNG, which is assumed from chemical considerations also to be monofunctional. In the studies of Fox and Fox (1967) the cells are from a different species, mouse, and are maintained in suspension, both for treatment with MMS and for cultivation, as opposed to the monolayers of the present study. A difference in cell membrane permeability might account for this decreased toxicity of MMS in comparison to MNNG. Furthermore, the <u>in vitro</u> alkylating activity of MNNG and MMS is compared by Lawley (1968), who found a greater extent (approximately three times) of methylation of adenine and guanine in DNA reacted with MMS at 0.2 M, than with MNNG as a saturated aqueous solution. As Singer and Fraenkel-Conrat (1967) report that the limit of solubility of MNNG in water at 25^{o} is about 0.04 M, the difference in degree of methylation may be due to the dose of exposure. The possibility that MNNG toxicity may be due to a reaction other than methylation of nucleic acid bases is considered later (III, D.3.b.iv.).

A twentyfold difference in ID_{50} is evident between the two nitrosamines, MNNG and N-methyl-N-nitrosourea (MNU), both of which are tested on cells derived from the same line (Don, Chinese hamster fibroblasts). In the studies of Sanders and Burford (1967) the cells are also cultured in monolayer but are treated with MNU in suspension, after trypsinisation. Cells harvested with trypsin from growth as a monolayer become rounded up as they detach from the substrate surface. It is possible that membrane permeability of such cells is altered and, here also, might account for the decrease in cytotoxic effect. Another factor which may be considered is the apparent half-life of the compounds, as McCalla, Reuvers and Kitai (1968) report that the half-life of MNU in phosphate buffer at pH 7 is approximately one fifth of that for MNNG

Agent*	Exposure time (hours)	$1D_{50}$ (μM)	Ref.
HS2	½	0.3	1
MNNG	1	15	2
MMS	3	100	3
MNU	2	370	4
DMN	2	insensitive	4

Table 17. Cytotoxicity of different alkylating agents for mammalian cells

The concentration (μM) which reduces survival to 50% of control ($1D_{50}$), at a given period of exposure, is shown for a bifunctional alkylating agent (HS2) and four nitrosamine compounds.

*See list of abbreviations.

1. Crathorn and Roberts, 1965.
2. Present study.
3. Fox and Fox, 1967.
4. Sanders and Burford, 1967.

examined under the same conditions.

Insensitivity of hamster cells to DMN is believed (Sanders and Burford, 1967) to be due to the absence from the cells of enzymes required to produce the active biological agent (Magee and Schoental, 1964; Magee and Barnes, 1967).

c. The morphology of colonies derived from MNNG treated cells

In the present study no colonies were observed with the gross morphological alterations reported by Sanders and Burford, both in hamster fibroblasts (1967) and other mammalian cells (1968). Nevertheless, a moderate disorientation of cells was noticed, at an early stage of incubation, at the periphery of a proportion of colonies derived from cells treated with MNNG (Fig. 21a). Here, also, differences of technique, involving the method of treatment, discussed above, and of plating the cells, may influence the response of the cells. Feeder layers of cells, X-irradiated with a lethal dose, were used in the present study only, the intention being to standardise conditions for cell inocula of differing viability. Notwithstanding, the X-irradiated cells may have influenced the growth of cells previously treated with MNNG in a manner similar to the proposals made for an effect by contiguous cells on the growth of neighbouring cells in vivo (Bullough, 1967) and in vitro (Friedman, Seegmiller and Subak-Sharpe, 1968). The presence of this peripheral disorientation and its regression with longer incubation could be explained by postulating the existence of a "controlling" factor, provided by the X-irradiated feeders for the cells derived from early divisions of cells treated with MNNG. Death of the X-irradiated cells would lead to a deficiency of this controlling factor, resulting in cellular disorientation, particularly in the cells growing out at the periphery. With prolonged incubation the concentration of this factor could approach the necessary level.

In their study of the morphological conversion of colonies of cells treated with MNU, Sanders and Burford (1967) also include experiments of inoculation of treated cells into animals. Tumours were induced in 100% animals inoculated with cells treated with 1 mM MNU, and in 50% animals inoculated with cells treated with 0.1 mM MNU. The significance of this impressive correlation, between the in vitro treatment of cells with a chemical carcinogen and tumour induction in animals by descendants of the treated cells, is diminished by the absence of results for cells which show similar conversion of colony morphology after treatment with methylurea (MU). MU is not known to have carcinogenic properties. It is perhaps pertinent, in evaluating the significance of tumour induction by cells treated with MNU, to note that the cell inoculum employed by Sanders and Burford (1967) is one hundredfold greater than that reported by Stoker (1963) to induce tumours by inoculation of hamster fibroblasts "transformed" by polyoma virus.

Alterations to the growth rate of cultured cells treated with DMN or MNU is also reported by Huberman, Salzberg and Sachs (1968). The authors did not find any colonies with a piled-up random pattern of growth a short time after treatment. Cells of later passages of the DMN treated populations, however, did produce colonies of this type and also induced tumours when large cell numbers ($5x10^6$) were injected subcutaneously into animals. The authors relate these characteristics of colony morphology and tumour induction to a secondary change in cells with an increased life time.

To substantiate speculations on the nature of the change observed in cells treated with MNNG, further studies would be required. These would include the characterisation of growth rate and colony morphology shown by treated cells and also the inoculation of treated cells into animals.

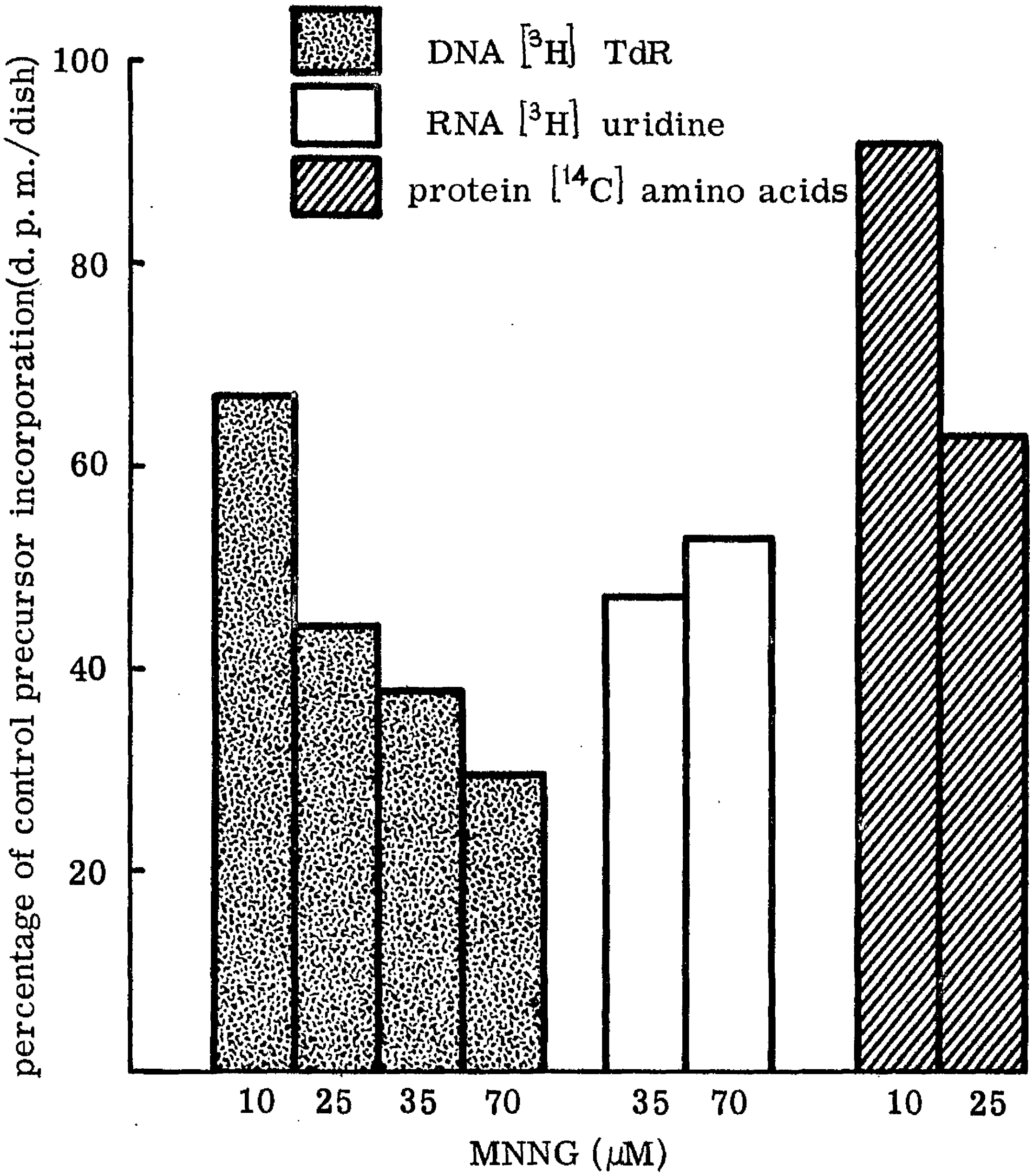
2. Inhibition of macromolecular synthesis.

The incorporation of the respective radioactive precursor into DNA, RNA or protein was inhibited in cells after treatment with MNNG (Fig. 40). Only the inhibition of incorporation of radioactive precursor into DNA was examined more closely, however. Before these results are

Figure 40. Inhibition of metabolism in cells exposed to MNNG.

The incorporation of the respective radioactive precursor into DNA, RNA or protein of cultured cells was assessed in separate experiments, as described in the text. The radioactivity/dish is shown as the percentage (mean of triplicates for DNA and RNA, of duplicates for protein) of incorporation by controls. Assessment of incorporation was made in the period immediately following addition of MNNG to the medium; the initial concentration of MNNG (µM) is given below the appropriate column.

The sensitivity of DNA synthesis to MNNG treatment is apparent.


DNA [^{3}H] TdR
RNA [^{3}H] uridine
protein [^{14}C] amino acids
percentage of control precursor incorporation(d. p. m./dish)
100
80
60
40
20
10 25 35 70 35 70 10 25
MNNG (μM)

considered it is important to outline the experience of other workers, according to published data of inhibition of DNA, RNA and protein synthesis, with MNNG or similar agents.

a. Alkylating agents as inhibitors of macromolecular synthesis

Crathorn and Roberts (1965) report a depression of DNA synthesis to 50% of untreated controls, as judged by incorporation of radioactive precursor, in mouse lymphoma cells treated with sulphur mustard (HS2) at a concentration sufficient to kill 90% of the cells. In later work these authors present evidence that a repair process, involving excision of alkylated deoxyribonucleosides, is operative in a line of HeLa cells treated with HS2 (Crathorn and Roberts, 1966; Roberts, Crathorn and Brent, 1968). The HeLa cells possess the same sensitivity to HS2 as the resistant mouse lymphoma cell line used in previous studies (Crathorn and Roberts, 1965). Cytotoxicity by HS2 is related by Crathorn and Roberts (1966) to the saturation of the cells' repair mechanisms. These authors calculate that alkylation of 1 in 10^6 nucleotides is sufficient to saturate the repair process in the mouse lymphoma cells sensitive to HS2. In the same cells, extensive killing (more than 90%) would follow alkylation of 1 in 10^5 nucleotides, of which 25% would involve crosslinkage of the DNA strands. Interference with DNA strand separation during replication would, therefore, inhibit DNA synthesis, and account for the decreased incorporation of radioactive precursor into DNA by sensitive mouse lymphoma cells, observed previously (Crathorn and Roberts, 1965). It is significant that RNA synthesis, as judged by incorporation of $[^3H]$uridine, is not impaired, in the sensitive mouse lymphoma cells, over the range of HS2 concentrations tested, namely, 1-5 μM (Crathom and Roberts, 1965).

Fox and Fox (1967) also employ mouse lymphoma cells to study the effect of treatment with the monofunctional agent, MMS. These authors record an inhibition of incorporation of radioactive precursor into DNA, RNA and protein; the inhibition of incorporation into DNA is the most severely affected. This pattern of inhibition is in keeping with the general experience of the biochemical effects of alkylating agents, as reviewed by Wheeler (1967) and is similar to the observations of the present study. In their discussion of the results, Fox and Fox (1967) suggest that alterations to the transport of precursor across the cell wall membrane, and within the cell, might contribute to the inhibition of incorporation of radioactive precursor into DNA, RNA or protein. This effect would be additional to the effects of alkylation of the nucleosides of DNA and RNA.

b. Nitrosamines as inhibitors of macromolecular synthesis

Magee and Schoental (1964) classify the nitrosamines into two groups, as follows:-

1. Those which are stable to alkali, require enzymatic activation and do not react with sulphydryl groups.
2. Those unstable to alkali and which interact at neutral pH with sulphydryl groups.

The studies relating to the biological properties of dimethylnitrosamine (DMN), a representative of the first group, are considered first. Experience of other workers with 1, 3-bis(2-chlorethyl)-1-nitrosourea (BCNU), an analogue of MNNG, and both belonging to the second group, is discussed next. The reports of the effects of MNNG on other experimental systems are then commented upon.

(i) Dimethylnitrosamine. Reports of the toxic (Barnes and Magee, 1954) and carcinogenic (Magee and Barnes, 1956) properties of DMN are followed by an account of the inhibition of protein synthesis, in the livers of rats, three hours after the animals were fed DMN (50 mg./kg.) by stomach tube (Magee, 1958). Emmelot and Benedetti (1961), in their study with the electron microscope of the changes in liver cells of rats after they were injected intravenously with DMN (50 mg./kg.), provide evidence that DMN induces structural disorganisation to the intracellular sites of protein synthesis. At three hours after injection there was swelling of the endoplasmic reticulum and detachment of the ribonucleoprotein particles. Villa-Trevino (1967) reports the analysis by zonal centrifugation of the post-mitochondrial supernatant obtained from the homogenised livers of rats injected intraperitoneally with DMN (30 mg./kg.). The results suggest that a breakdown occurs, of ribosomal aggregates (microsomes) into smaller units. $[^{14}C]$Radioactivity is detected in various RNA and protein fractions from the liver cells following the injection of rats with $[^{14}C]$DMN. The greatest amount of $[^{14}C]$ radioactivity on ion-exchange chromatography of acid hydrolysates of nuclear RNA from livers of rats treated similarly is present in the fractions corresponding to 7-methylguanine. The author believes that microsomal disruption, and possibly also impaired activity of methylated mRNA to direct the incorporation of amino acid into polypeptide, might explain the decreased incorporation of $[^{14}C]$leucine into liver proteins of rats treated with DMN, which he reports (Villa-Trevino, 1967).

It is apparent from the above studies of the biological activity of DMN that emphasis is placed on the inhibition of protein synthesis. Incorporation of precursor into RNA of rat liver slices is examined by Hultin, Arrhenius, Löw and Magee (1960), who find that the incorporation of $[^{14}C]$adenine into RNA is reduced in liver slices preincubated with 1 mM DMN, but is inhibited less than is the incorporation of amino acid into protein.

Another aspect of the biological reaction(s) of DMN is the ability to methylate nucleic acids (Magee and Farber, 1962; Lawley, Brookes, Magee, Craddock and Swann, 1968), and also proteins, forming methylated histidine residues (Magee and Hultin, 1962). A comprehensive appraisal of the significance of the activity of the nitrosamines as alkylating agents is given by Magee and Barnes (1967). Some evidence that alkylation of nucleic acids may be of primary importance in the mechanism of carcinogenesis has already been mentioned (III, A.3.b.). Recently, a comparative study is reported (Swann and Magee, 1968) of the alkylation of nucleic acids of the rat by two N-methylnitrosamines and two methylating agents. The induction of tumours in rats by these agents is then examined, in the light of the data on quantitative and qualitative alkylation of DNA and RNA in different organs. The authors conclude that if alkylation is responsible for carcinogenesis by nitrosamines, DNA is not the primary target. The possibility is considered that alkylation of protein, RNA or some other cellular constituent might interfere with the expression of genetic information.

The relationship of these observations on the biological effects of DMN to the present study of MNNG with cultured cells is uncertain. The two compounds differ widely in their chemical reactivity, stability and solubility. DMN, a member of the first group of nitrosamines, as classified at the beginning of this section, (b), is freely soluble in aqueous solvents and is rapidly distributed uniformly throughout the body water after administration (Magee, 1956). MNNG, on the other hand, is a member of the second group and is more soluble in lipid than in aqueous media (Freese and Bautz-Freese, 1966). It is possible that the dissimilarity between DMN and MNNG, on chemical grounds, might be reflected in different intracellular localisation of the biologically active agent. This difference could be expressed in the pattern of metabolic inhibition, as here.

(ii) 1,3-Bis-(2-chlorethyl)-1-nitrosourea (BCNU). As a result of screening tests for anti-cancer activity among analogues of MNNG (Skinner _et al._, 1960) the compound BCNU was developed. The strong activity of BCNU against mouse intracerebral leukaemia is attributed by Schabel _et al_. (1963) to its free solubility in lipids. As BCNU is a representative of the second group of nitrosamines as classified above, it is of interest to compare the results of the present study with those of Gale (1965). This author examines the incorporation of radioactive precursor, into DNA, RNA or protein, by Ehrlich ascites tumour cells during the second hour after treatment with BCNU. The rate of incorporation of radioactive precursor into DNA and protein decreases, the more severely into DNA. The author's observation that the inhibition is without lag must be accepted, but fails to take into account events occurring during the hour of treatment. The incorporation of radioactive uridine into RNA of cells treated with 25 and 50 µM BCNU is increased over untreated controls. In the present study with MNNG the incorporation of $[^3H]$uridine by MNNG-treated cells shows a similar increase over untreated controls (Fig. 26). Here, the treated cells can be compared at all times with untreated controls at zero time since growth of the MNNG-treated cells is arrested by treatment with 25 µM MNNG or more (Fig. 17). The effect of exposure to BCNU on cultured cells is also studied by Wheeler and Bowdon (1965), with both solid and ascites forms of the mouse leukaemia cell-line, L 1210. The major effect observed is an inhibition of the incorporation of purine precursors into DNA and a greater inhibition of the incorporation of $[^{14}C]$ formate into nucleic acids than of $[^3H]$leucine into proteins. The inhibition of incorporation is discussed in relation to the available evidence for the generation from BCNU of a diazoalkane, as the active biological agent.

(iii) N-Methyl-N'nitro-N-nitrosoguanidine (MNNG). Since the effect of treatment with MNNG on the synthesis of DNA, RNA and protein by mammalian cells has not been studied until now, the results of the present study are compared with the data from studies of metabolic inhibition with MNNG in non-mammalian systems.

Terawaki and Greenberg (1965) examine the quantity of DNA, RNA and protein in aliquots removed from cultures of <u>Escherichia coli</u> one hour after treatment with 10 µg./ml. MNNG (approx. 70 µM), for comparison with untreated controls. A decrease of between 40-50% in the quantity of each macromolecule is recorded in the treated population; but so also is the yield of cells similarly decreased, which satisfactorily explains the greater bulk of the "inhibition".

The authors also report that treatment with MNNG (approx. 70 μM) reduces the activity of DNA as the agent of transformation, by about 50%, in a system employing Bacillus subtilis strains. Strains of Escherichia coli are also used by Cerdá-Olmedo and Hanawalt (1967) in a study of MNNG action. These authors attach great importance to the inhibition of incorporation of radioactive precursor into RNA or protein, and associate the ability to synthesise functional protein with survival of cells treated with MNNG (approx. 7 mM). Evidence of interaction with protein already synthesised is provided by studies of β-galactosidase activity in cells treated with MNNG (approx. 7 mM). Inactivation of enzyme is reported as following simple exponential kinetics with increasing time of treatment. However, ribosomes are considered as the principal targets for lethal activity of MNNG on the cells. It is also postulated that the mutagenic effect of MNNG could be due to the interaction of MNNG with the enzymes related to the replication of DNA. Support for the latter hypothesis is provided in a later report (Cerdá-Olmedo, Hanawalt and Guerola, 1968) on the ability of MNNG to induce mutations at the region of the bacterial chromosome undergoing replication. This work will be discussed later (III, D.3.c). An examination of the activity of MNNG with an in vitro protein synthesising system extracted from Escherichia coli is made by Chandra, Wacker, Süssmuth and Lingens (1967). Synthetic polymers of adenylic (poly A), cytidylic (poly C), uridylic (poly U) or of adenylic plus guanylic (poly AG) acids are treated with MNNG (1 mM) and then tested for ability to direct incorporation of radioactive amino acid into polypeptide. Quantitative comparisons are made of incorporated radioactivity from different amino acids with the same polyribonucleotide, treated and untreated. The alterations of incorporation, following MNNG treatment, indicate that methylation of nucleotide bases is probably responsible for the predominant effects, assuming the nucleotide triplet code for amino acids (see I, B.3.b.) to be correct. Nucleoside base change by deamination, involving, for example, the change cytosine to uracil, is not considered to be of significance. This observation has importance because nitrous acid, an agent of deamination of nucleoside bases (Freese, 1963), has been reported to evolve from MNNG at low pH (McKay and Wright, 1947) and has been proposed as the active agent of mutagenesis by MNNG at low pH (Zimmerman, Schwaier and Laer, 1965).

Chandra _et al_.(1967) support their belief, that the major effect on amino acid incorporation patterns is due to methylation of nucleoside bases, with studies of the relative amounts of radioactivity bound to synthetic polyribonucleotides treated with $[^3H\text{-methyl}]$ MNNG. Indeed, it has also been suggested (Villa-Trevino, 1967; Shank, 1968) that the early inhibition of protein synthesis following DMN treatment _in vivo_ may be due to methylation of mRNA.

The "enzyme" and "ribosome" fractions extracted from _Escherichia coli_, previously treated with MNNG (0.2 mM) are tested, by Lingens, Süssmuth, Wacker and Chandra (1967), as components, in combination and separately, of an _in vitro_ protein synthesising system. Only the "ribosome" fraction is found to show decreased activity, which is considered to result from methylation of ribosomal RNA. The authors also report that DNA treated with MNNG _in vitro_ shows a reduced ability to act as template in the (DNA dependent) RNA polymerase reaction (see I, B.2.). The degree of this effect is correlated with the extent to which the DNA is methylated, although it is also stated that interference with the mechanism of transcription itself is probable _in vivo_.

Experiments with _Euglena gracilis_, a species of the blue-green algae, treated with MNNG, are reported by Allan and McCalla (1967). The incorporation of $[^{14}C]$adenine into DNA is inhibited to a greater degree than into RNA; incorporation of $[^{14}C]$leucine into protein is least inhibited. The incorporation of radioactivity into nucleic acids is rapidly inhibited, but a delay of 45-60 min. elapses before the incorporation into protein is inhibited. Damage to DNA "primer" is believed to be the major factor causing these effects. Reference has already been made (III, A.2.b.) to the activity of MNNG as a chloroplast "bleaching" agent in Euglena gracilis. The mechanism of bleaching is not understood, but may be related to interaction of the agents with chloroplast DNA (McCalla, 1965). Studies of cross resistance patterns to other biological inhibitors, for example, ultra-violet light, in a mutant strain of Euglena gracilis indicate that lethal activity and the loss of chloroplasts, by MNNG-treatment, are not related to the same event (McCalla, 1967b).

c. Summary: inhibition of macromolecular synthesis

The results of the present study, in which incorporation of radioactive precursor is inhibited, in decreasing order of severity, into DNA, RNA and protein of mammalian cells, are similar to experience with monofunctional alkylating agents and with another nitrosamide, BCNU. They are in contrast to the sensitivity of protein synthesis in mammalian liver cells to inhibition by DMN, as judged by incorporation of precursor. In bacteria, however, the effect of MNNG on protein synthesis is more closely studied, both in vivo and in vitro, and appears to have significance to the observed biological effects.

No comment, however, can be made of the possible effects of MNNG on the synthesis of RNA and protein in mammalian cells as no further examination of this aspect was made.

3. Inhibition of precursor incorporation into DNA by MNNG.

The inhibition of precursor incorporation into DNA was examined in more detail because of:-

1. the potency of MNNG as a mutagen (see III, A.2.a. and b.)
2. the incorporation of radioactive precursor was inhibited to a greater extent into DNA than into RNA or protein (Fig. 40).

3. the evidence, from the dose-response of the inhibition of $[^3H]$thymidine incorporation in the first hour after treatment (Fig. 25), for a single intracellular target.

Possible reasons for the decreased incorporation of precursor into the DNA cells treated with MNNG include:-

a reduction in the number of cells synthesising DNA,

or a failure of precursor to penetrate within the cell,

or a rapid breakdown of newly-synthesised DNA,

or interference by the agent with the activity of enzymes responsible for the synthesis of deoxyribonucleotides and their polymerisation into DNA.

The "temperature" effect, causing inhibition of $[^3H]$thymidine incorporation by controls of the early experiments, discussed already (III, C.2.a.i.) is an example of the first possibility. It is still feasible that cells not subjected to any temperature shock are prevented by MNNG from passing from G_1 into S. This might have the effect of producing values for a linear dose-response relationship - due, for example, to inactivation of an enzyme - which extrapolates below zero inhibitor dose. Although the dose-response of $[^3H]$thymidine incorporation in the present study has this appearance (Fig. 25), it is probable that other factors also contribute to this effect (see III, C.2.a.iii.).

DNA synthesis is reported to be inhibited in cultured cells after treatment with a number of agents, for example,

(i) mitomycin C, in bacteria (Sekiguchi and Takagi, 1960) and in rabbit kidney cells (Adams, Abrams and Lieberman, 1965),

(ii) hydroxyurea, in rabbit kidney cells (Adams, Abrams and Lieberman, 1966), and

(iii) high concentrations of deoxyadenosine in Ehrlich ascites cells (Klenow, 1959).

Possible explanations of the mechanism of action of these inhibitors are suggested, from other studies, to be,

(i) for mitomycin C, the cross-linkage of DNA strands (see Waring, 1968),

(ii) for hydroxyurea, the inhibition of ribonucleotide reductase (Adams and Lindsay, 1967; Elford, 1968), and

(iii) for deoxyadenosine, an allosteric inhibition of ribonucleotide reductase by deoxyadenosinetriphosphate (Larsson and Reichard, 1966) which accumulates in cells treated with high concentrations of deoxyadenosine (Klenow, 1962).

In the present study, the incorporation of 20% (v/v) NCTC 109 in the growth medium ($PNFC_{20}$) results in the presence, at approximately 10 µM each, of the four deoxyribonucleosides, deoxyadenosine, deoxycytidine, deoxyguanosine and (deoxy)thymidine. For this reason it is assumed that inhibition of either the enzymes required for purine and pyrimidine synthesis, or the reductase enzymes - for conversion of ribonucleotides to deoxyribonucleotides - would not constitute a major factor in the reduction of precursor incorporation into DNA. For this reason, only two enzyme pathways were examined, namely, (1) the thymidine and thymidylate kinases, and (2) DNA polymerase, the former in vivo and the latter in vitro.

a. Kinase activity in cells treated with MNNG

The penetration of $[^3H]$thymidine into the cells and activity of the thymidine and thymidylate kinase enzymes is not significantly altered by treatment of cells with MNNG, in comparison with untreated controls (Fig. 28). This examination is insensitive, however, and should be supported by information of nucleotide pool size. In the present experimental system the acquisition of data for interpretation of nucleotide pool size is complex. The decision to examine the effect of MNNG on DNA polymerase reaction was, therefore, taken.

b. Activity of DNA polymerase extracted from hamster cells

The inhibition of DNA polymerase activity, in vitro, by MNNG is best considered under four headings in an assessment of its mechanism. These are:-

(i) protection of DNA polymerase against inhibition by MNNG,

(ii) rate of inactivation of DNA polymerase by MNNG and the effect of other inhibitors,

(iii) DNA polymerase kinetic studies, with and without inhibition by MNNG.

(iv) the biological agent derived from MNNG.

The results may then be evaluated in relation to the in vivo experiments.

(i) Protection of DNA polymerase against inhibition by MNNG. Reduced glutathione (GSH), equimolar with MNNG, reduces the inhibition of the DNA polymerase reaction with MNNG by more than 40% (Fig. 35), whereas 2-mercaptoethanol, at five times the molarity of MNNG, reduces by just less than 30% (Fig. 32), when either of these compounds is included in the preincubation solution of enzyme extract and MNNG. The reduction is calculated here by comparison of results obtained when enzyme extract is incubated with MNNG in the presence or absence of exogenous thiol groups. A similar activity of GSH and 2-mercaptoethanol in the protection of DNA polymerase, extracted from Landschutz ascites tumour cells, prompts Keir and Shepherd (1965) to conclude that thiol group(s) of the enzyme are probably the primary site(s) of action of the inhibitors used, namely, p-hydroxymercuribenzoate and sarkomycin. The possibility that thiol groups of hamster (Don cell line) DNA polymerase may be the targets of interaction with MNNG, or a breakdown product, must be interpreted with caution.

Thiol groups in enzyme proteins serve the function of determining tertiary molecular structure and only a very few are expected to be involved in the catalytic process (Boyer, 1959). The interpretation of protection by thiols against inhibitors which themselves react with thiol groups, as is expected to be the case for MNNG (see III, A.3.a.) is not simple as the result may reflect only a reduction in the effective concentration of inhibitor (Webb, 1966). MNNG reacts strongly with amines (see III, A.1.), yet lysine, a diamino acid, failed at even twice the molarity of MNNG to protect against inhibition (Fig. 34). However, the competition factor of free thiol groups is believed to be 1,000 times higher than that of terminal amino groups (see Schoental, 1966a) and may explain this difference of effect. It is also possible that the reduction of MNNG inhibition by GSH is due to a considerably reduced biological half-life. In the absence of comparative data for the half-life of MNNG in enzyme solutions which contain GSH or lysine it is proposed that MNNG can interact with enzyme thiol(s).

The protection afforded to DNA polymerase by the presence of substrate DNA in the enzyme extract solution incubated with MNNG (Fig. 36) suggests that binding site(s) on the enzyme for substrate DNA might be target site(s) for inactivation by MNNG. As pointed out by Webb (1966) it is also necessary to consider alternatives, namely, that reaction of inhibitor at adjacent sites may alter the affinity of the "active centre" for substrate, or that binding of substrate may so stabilise enzyme structure as to impair reaction of other sites with inhibitor. On the other hand, substrate deoxyribonucleoside triphosphates, at the standard concentration (50 mμmoles per assay) failed to alter the inhibition of DNA polymerase by MNNG, when they were included in the preincubation solution. This concentration gives a molar ratio, of inhibitor to deoxyribonucleoside triphosphate, of 5, which might not favour successful competition for active sites. Kimball and Wilson (1968), however, obtain protection against the inhibition of Ehrlich ascites tumour cell DNA polymerase reaction <u>in vitro</u> by β-D-arbinosylcytosine, with deoxycytidine-5'-triphosphate at a corresponding ratio of 2.86. In view of the unexplained inhibition of activity of untreated control enzyme preincubated with the triphosphates in the present study, no comment can be made on the possibility of reaction of inhibitor with the binding site(s) of enzyme for deoxyribonucleoside triphosphate.

The amino acids histidine or lysine, at 2 mM in the preincubation solution of enzyme extract and MNNG, failed to alter the inhibition. These amino acids are believed to be associated with the active centre of enzymes (see Dixon and Webb, 1964c) as has been demonstrated, for example, in the case of lysine, for ribonuclease

(Klee and Richards, 1957). Indeed, Erhan (1968) proposes that one unit of DNA polymerase is rich in lysyl residues. The enzyme, according to this author, comprises three units. of which the one rich in lysyl residues, and described as a "wedge", recognises the starting region of the chromosome for DNA replication. The wedge also holds the two active enzyme units together with a particular geometry. The reaction involved in the inhibition of DNA polymerase activity is likely to be complex. The crude nature of these experiments makes it impossible to exclude a significant interaction of MNNG, or a breakdown product, with lysyl or histidyl residues present in the DNA polymerase molecule.

(ii) Rate of inactivation of DNA polymerase by MNNG and the effect of other inhibitors. The initial rapidity of inactivation with gradual decline (Fig.30b) may be a function of the variation in reactivity of protein thiol groups (Barron, 1951) and/or the rapid rate of MNNG hydrolysis at the pH of the solution (see III, B.2.). The failure to reverse the inhibition of enzyme by the addition of reduced glutathione at the end of preincubation (Fig. 35) indicates that denaturation, or perhaps dissociation of enzyme units, may have occurred. Also, dissociation of co-factor, for example, Mg^{2+}, cannot be excluded. Nevertheless, guanidine alone, reduces the activity of DNA polymerase by more than 25% (Fig. 32). Since this compound is known to denature proteins (Groves, Hipp and McMeekin, 1951) an inhibition might be expected. The greater inhibition by MNNG suggests that denaturation alone would not account for the inhibitory activity.

In the present study, the inhibition of hamster (Don cell line) DNA polymerase by p-chloromercuribenzoate (20 μM) is of the order of 25% (Fig. 32). This value is considerably less than the 90% inhibition of Landschutz ascites tumour cell DNA polymerase reaction by p-hydroxymercuribenzoate at equivalent concentration (Keir and Shepherd, 1965). It is possible that a difference in penetration, due to steric or electrostatic factors, of the active agent within the enzyme accounts for the disparity of inhibition.

(iii) DNA polymerase: kinetic studies. Further information on the mechanism of DNA polymerase inhibition by MNNG, in vitro, is given from appraisal of the results of enzyme kinetic studies. The activity of DNA polymerase extracted from untreated cells and assayed according to standard conditions with varied concentration of substrate DNA is shown in Fig. 37a, as the double-reciprocal plot according to Lineweaver and Burk (1934). A linear correlation between 1/V and 1/S is deduced by these authors from theoretical considerations for enzyme reactions obeying simple Michaelis-Menten kinetics, according to the equations:-

$$E + S \longrightarrow ES$$

$$ES + \text{reactant} \longrightarrow E + \text{product}$$

where E, S and ES represent the total concentrations of enzyme, substrate and the active complex, respectively, and V is the rate of the reaction, normally given as the amount of product formed. A deviation from Michaelis-Menten kinetics for the hamster DNA polymerase is evident from the parabolic nature of the graph (Fig.37a). Curves of this type are expected, from theoretical considerations (see Dixon and Webb, 1964a) for:-

(i) an enzyme which acts upon two substrates, or

(ii) an enzyme which is activated by substrate.

The theoretical reactions from which the equations for such curves are derived are shown below (after Dixon and Webb, 1964a).

(i) $E + S_t \rightleftharpoons ES_t$, Dissociation constant $= K_{St}$

$E + S_{DNA} \rightleftharpoons ES_{DNA}$, Dissociation constant $= K_{S_{DNA}}$

$ES_t + S_{DNA} \rightleftharpoons ES_tS_{DNA} \longrightarrow E + P$, Dissociation constant $= K'S_t$

$ES_{DNA} + S_t \rightleftharpoons ES_{DNA}S_t \longrightarrow E + P$, Dissociation constant $= K'S_{DNA}$

(ii) $E + S_t \rightleftharpoons ES_t$, Dissociation ~~constant~~ (inactive) $= K_{St} = \infty$ (inactive)

$E + S_{DNA} \rightleftharpoons ES_a$, Dissociation constant $= K_{Sa}$ = apparent Michaelis constant

$ES_a + S_t \rightleftharpoons ES_aS_t \rightarrow E + P$, Dissociation constant $= K'_S$ = true Michaelis constant

Where E, S_t, S_{DNA}, ES_t, ES_{DNA}, ES_a and P represent the concentrations of enzyme, deoxyribonucleoside triphosphate, DNA primer, the inactive enzyme complex with deoxyribonucleotide triphosphate or with DNA primer, the active enzyme complex and the product formed, respectively. It is assumed that there is only one binding site on the enzyme for each substrate.

The in vitro DNA polymerase reaction employed in the present study utilises denatured, single-stranded DNA as primer, in contrast to the native, double-stranded DNA which is replicated in vivo. The two substrates, according to the first proposal above, would be either (a) the monomers, or (b) the single-stranded polymer, of phosphorylated deoxyribonucleosides, although here no distinction is made between the different mononucleotide units. For the second hypothesis given above it is proposed that binding of substrate DNA is required to activate the enzyme. From the results of the present study, however, it is not possible to distinguish between these alternative enzyme reactions.

The double-reciprocal plot of values obtained with DNA polymerase inhibited by MNNG (Fig. 38) shows an alteration from the control treated enzyme, which permits speculation on the nature of the inhibition. For an enzyme acting on two substrates the slope of the curve may be altered by changes in the dissociation constants (K_{St} or $K_{S_{DNA}}$) of either substrate with the enzyme. In the case of an enzyme activated by its substrate, similar changes follow variation of the dissociation constant, K_{Sa}, of the active enzyme complex.

However, it is also possible that the relationship of the reciprocals of reaction rate and substrate (DNA) concentration, for DNA polymerase treated with MNNG, is linear (Fig. 41). Such a change, from parabolic to linear, in the case of an enzyme activated by substrate, is given in the theoretical example of Dixon and Webb (1964a) where K_{St} (or equivalent) ceases to be infinite.

Thus, for either of the proposed models, (i) or (ii) above, of DNA polymerase action, there is evidence from kinetic studies for an altered affinity (reflected in K, the dissociation constant) of enzyme for <u>both</u> substrates, DNA and deoxyribonucleoside triphosphate. Although from these studies it is not possible to distinguish conclusively any one of these factors to be of greater significance in the inhibition of enzyme by MNNG, the evidence is in favour of an altered affinity of the enzyme for single-stranded DNA, in view of its "protective" capacity noted above (III, D.3.b.i., see Fig. 36).

When the rate of DNA polymerase reaction inhibited by MNNG is expressed as a function of MNNG concentration, the relationship is again non-linear (Fig. 39). This may be interpreted to represent a "mixed" mode of inhibition, where the reaction is both partially competitive, affecting affinity for substrate through reaction with neighbouring groups, and non-competitive. For such inhibitors it is not possible to calculate an inhibitor constant, K_i, by graphical procedures alone (Dixon and Webb, 1964b).

Interpretation of kinetic studies of both enzyme action and its inhibition by MNNG is complicated by the knowledge that:-

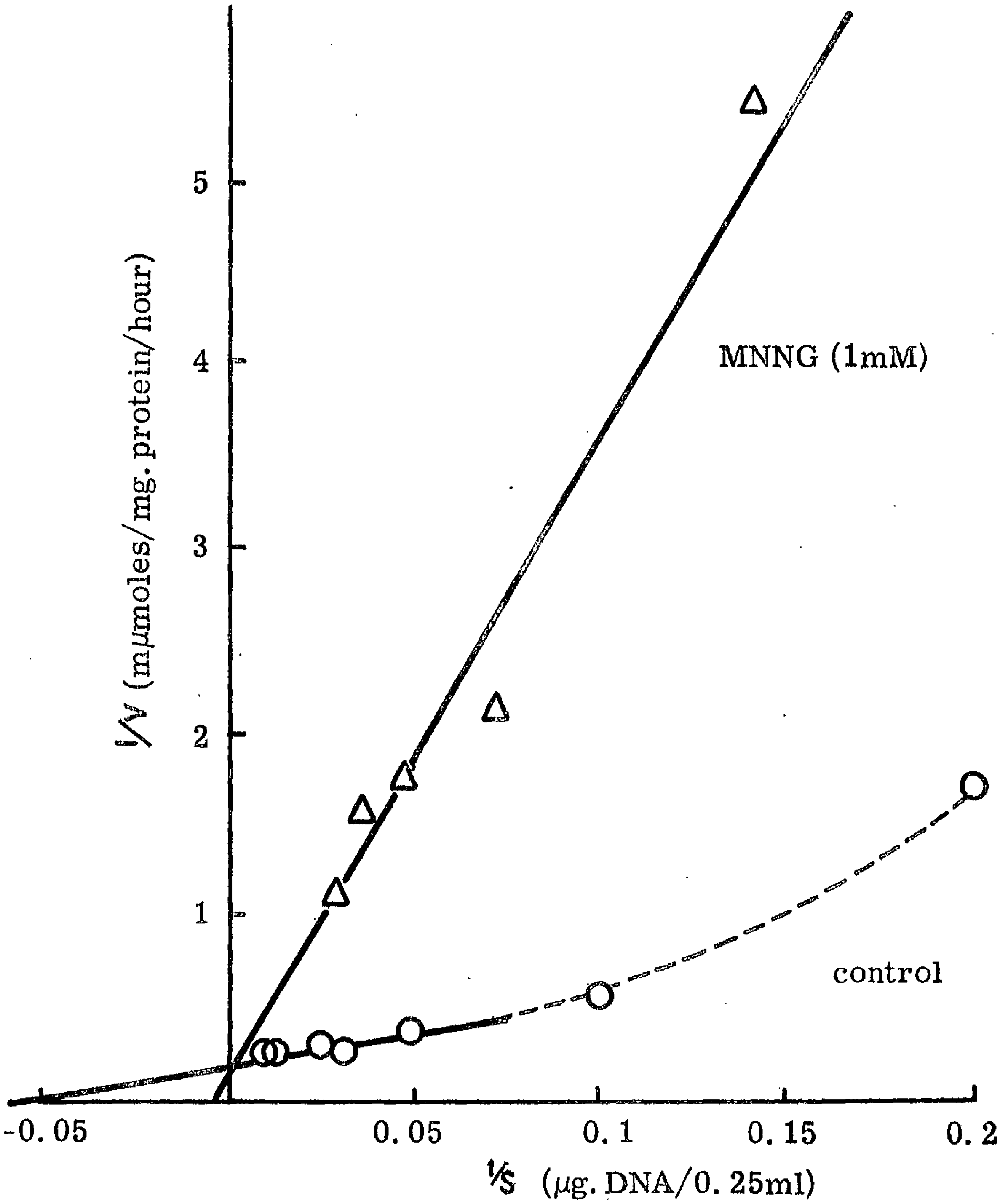
(i) no differentiation is made between the "end-addition" and "replicative" enzyme activity in the cell extract (see III, A.4.e.),

(ii) the cytoplasmic extract of the present study possesses activity with native DNA, which amounts to approximately 15% of total activity (Fig. 33),

(iii) the enzyme reaction begins to decline when high concentrations of denatured DNA are employed as substrate (Fig. 29a).

Figure 41. Inhibition of DNA polymerase activity by MNNG: reaction kinetics.

The results of Figure 38 (page 78) are illustrated as a linear double-reciprocal plot for the MNNG inhibited reaction. The significance is discussed in the text.



MNNG (1mM)
control
1/v (mμmoles/mg. protein/hour)
1
2
3
4
5
-0.05
0.05
0.1
0.15
0.2
1/S (μg. DNA/0.25ml)

Approximate values of the apparent Michaelis constant (K_m) for enzymes which show double-reciprocal plots of the character observed in the present study, can be calculated from the points of intersection on the abscissa, of tangents to the curve, as 1/S tends to zero (Dixon and Webb, 1964a). However, due to the factors outlined immediately above, that make a meaningful assessment of hamster DNA polymerase reaction difficult at present, it is concluded that a more purified enzyme preparation is required for more definitive information regarding the mechanism of enzyme inhibition by MNNG, and for the calculation of enzyme constants.

(iv) MNNG: the biologically active agent. It is generally believed that the biologically active nitrosamines owe their activity to the generation from them of diazoalkanes, which cause the alkylation of nucleic acids and proteins (Magee and Shoental, 1964; Magee and Barnes, 1967; Druckrey *et al.*, 1967). Indeed, diazomethane, the diazoalkane derived from MNNG (McKay, 1948) and a potent agent of nucleic acid methylation, *in vitro*, (Kriek and Emmelot, 1964) is proposed as the biologically active agent derived from MNNG that is responsible for both lethal and mutagenic events in bacteria (Cerdá-Olmedo and Hanawalt, 1968).

From acid hydrolysates of DNA treated with MNNG *in vitro* Craddock (1968) and McCalla (1968a) isolate 7-methylguanine. Lawley (1968) isolates both 7-methylguanine and 3-methyladenine, the products expected on theoretical grounds (Lawley, 1966) from hydrolysates, at neutral pH, of DNA treated with an alkylating agent. Methyl carbonium ion, by analogy with the proposed mechanism of hydrolysis of N-methyl-N-nitrosourethane (MNUE) in aqueous solution, would be the methylating agent according to Lawley (1968). Craddock (1968), however, considers that the reaction may occur according to the proposals of Henry (1950) for the reaction of MNNG with amines:

(i) MNNG + $R\text{-}NH_2$ ——> R-NG + (monomethylnitrosamine)

(ii) (monomethylnitrosamine) + guanine reside ——> 7-methylguanine.

Craddock believes that any guanido compounds (R-NG) formed, by reaction of MNNG with alkyl- or arylamines ($R-NH_2$) would probably have been destroyed by the acid hydrolysis treatment of the DNA required to release the alkylated units.

McCalla (1968a) observes that the presence of cysteine in the reaction mixture increases the amount of radioactivity bound to DNA reacted with $[^{14}C\text{-methyl}]$ MNNG and that the major radioactive component has the characteristics of 7-methylguanine. On the other hand, the presence of cysteine reduces the amount of radio-activity that becomes bound to DNA reacted with $[^{14}C\text{-guanidine}]$ MNNG. This reduction in the extent reaction (guanidination) by cysteine is similar to the protection of DNA polymerase by thiols, in the present study, against inhibition <u>in vitro</u> by MNNG. McCalla does not identify the component carrying the $[^{14}C\text{-guanidine}]$ radioactivity, however.

In later work McCalla and Reuvers (1968) report on the products obtained by enzymatic hydrolysis of ovalbumin after its reaction <u>in vitro</u> with MNNG. The lysine-arginine ratio in the constituents of the hydrolysate is altered in a manner consistent with a decrease in lysyl residues. Also, a compound with the characteristics of nitrohomoarginine, the predicted product of reaction between lysine and the guanido group of MNNG , is isolated. Reaction of the guanido group with protein thiol residues is not considered by the authors to constitute a significant factor in the reaction. Nevertheless, as thiol groups, reported as masked in ovalbumin (Greenstein, 1938) are important in the structural integrity of molecule, reaction at these sites (thiols) may have greater consequence on enzyme activity than at lysyl residues. Indeed, Klee and Richards (1957) find that guanidination of up to nine of the lysyl residues of bovine pancreatic ribonuclease does not affect the activity of the enzyme.

The products of reaction between cysteine and MNUE are reported by Schoental (1966a) and discussed in relation to the possible effects on catalytic activity of enzymes. The author also considers that in the intact cell the proximity of protein thiol groups may be a factor determining the reaction of particular regions of the DNA of the chromosome with the active agent derived from the nitrosamine. This latter hypothesis is made with particular reference to the carcinogenic properties of MNUE. Schoental comments on the ability of MNNG also to react with thiols, at the time of reporting the potent activity of MNNG as a carcinogen (Schoental, 1966b).

In discussing the possibility of reaction of MNNG with thiols it should be mentioned that the importance of a nitroso group of high electron density, in relation to biological activity of the nitrosamines, is pointed out by Arcos and Arcos (1962). According to the proposal of Lawley (1968) for MNUE, the electron shift on hydrolysis of MNNG in aqueous solution might be as shown in Fig. 42. The increase in the positive charge of the guanido-carbon would favour its reaction with ionised S^{-}.

Wheeler and Bowdon (1968) propose that isocyanate, generated from BCNU, accounts for the ability of this nitrosamide to inhibit the DNA polymerase activity in extracts from mouse leukaemia cells. McKay (1952) states that MNNG is capable of generating isocyanate, but not at physiological temperature. It is therefore unlikely that isocyanate is the active agent in the present study, in the cells or <u>in vitro</u>. Organic peroxides are believed to be possible agents of MNNG activity (Gichner, Michaelis and Rieger, 1963) but the inability of catalase to protect against inhibition of DNA polymerase (Fig. 34) does not support this theory as an explanation of the <u>in vitro</u> effects.

The intercalation of small molecules between the constituent bases of the DNA helix might be expected to have profound effects on cellular function, as discussed, in the case of proflavine, by Waring (1968). Singer and Fraenkel-Conrat (1967) propose that

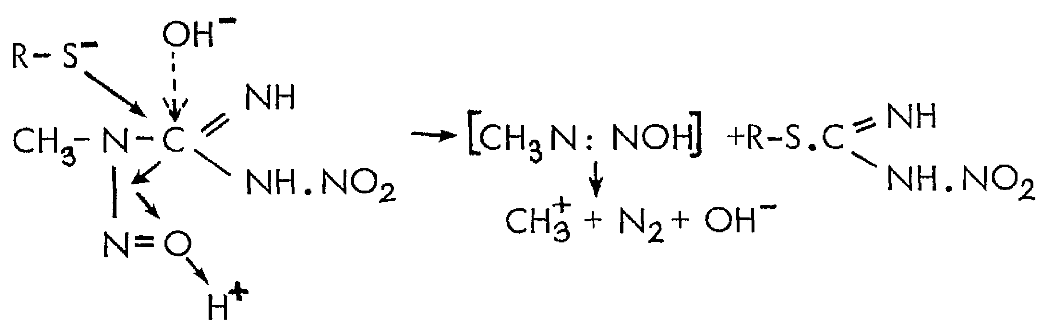


Figure 42. Diagrammatic representation of MNNG hydrolysis in aqueous solution, and possible reaction with ionised thiol (R-SH) (after Lawley, 1968; direction of electron flow is arrowed).

intercalation of MNNG between nucleotides may account for the susceptibility of guanine, in tobacco mosaic virus RNA, to react with MNNG. No experiments were made in the present study to examine for the phenomenon of intercalation, either in treated cells or in the in vitro system.

Mandell and Greenberg (1960) propose that the whole molecule of MNNG is the biologically active agent, but in view of the evidence given above of the rapid decomposition of, and production of reactive groups from, MNNG at physiological pH, this possibility is considered unlikely.

It is concluded, therefore, that in vitro DNA polymerase activity is inhibited by MNNG through guanidination of enzyme groups, most probably thiols. It is also believed that guanidination of thiol or lysyl (or other animo group) residues of the cellular constituents plays a significant part in biological activity of MNNG.

c. Inhibition of precursor incorporation into DNA: a summary

It remains to be established that inhibition of DNA polymerase activity by MNNG in vitro explains the inhibition by MNNG of $[^3H]$ thymidine incorporation into DNA of the cells. Of the reasons given above (3.), inhibition of enzymes involved in replication of DNA is believed to play a significant part in vivo. Evidence that DNA polymerase is the target of inactivation in cells in vivo is provided by the dose-response of inhibition, which is of single-hit type, the persistence of kinase activity in MNNG treated cells, and, in addition, the inhibition by MNNG of the DNA polymerase reaction in vitro. The assumptive nature of this evidence has already been discussed and it fails to take into account the possibility that MNNG may act as a general enzyme inhibitor in vitro.

The twentyfold difference between the molarities of MNNG that cause equivalent in vivo or in vitro inhibition of precursor incorporation may be used in argument against the correlation of the two results. However, concentration of MNNG in particular intracellular sites may occur, and lead to greater molarities than in the growth medium. In view of the lipophilic nature of MNNG, such concentration may indeed take place in the intracellular lipids; cell membranes, which have a rich content of lipids, are potentially favourable sites. The significance of membranes to DNA replication in mammalian cells is appreciated from the autoradiographic evidence of Comings and Kakefuda (1968) that DNA replication in cultured human cells is initiated at the nuclear membrane. Active DNA polymerase may be membrane associated, if not bound, in the cell, and therefore be rendered particularly susceptible to interference with MNNG, or a breakdown product.

Although it appears that guanidination of enzyme groups is a factor responsible for _in vitro_ inhibition of DNA polymerase activity by MNNG, the rational behind this reaction follows from observations of MNNG instability in aqueous solvents at different hydrogen ion concentrations, for example. The stability of MNNG in lipids is not known, and furthermore, molar concentrations (locally) of thiol or amino groups might also be expected to influence the reactivity of MNNG within the cell. In this context it is interesting to note that one possibility considered by Gichner and Veleminsky (1967) to explain the mutagenic activity of MNNG for _Arabidopsis_ seeds, in contrast to the absence of activity for barley seeds, is the difference in metabolism of the seeds. _Arabidopsis_ seeds utilise lipids whilst the seeds of barley utilise starch.

Replication of the chromosome is a membrane associated process in bacteria also (Marvin, 1968). In _Escherichia coli_ strains Cerda-Olmedo, Hanawalt and Guerola (1968) demonstrate the ability of MNNG to induce mutations in the chromosome at the region of replication. The mutant yield for different markers is examined in synchronised cultures exposed (30 min.) to MNNG at intervals during growth. A map of marker frequency, related to the mean generation time of the cells, corresponds closely to the genetic map of the chromosome obtained by conventional techniques of genetic recombination (e.g. transformation). The authors believe that the conformation of the DNA strand (single as opposed to double) at the replication point increases the susceptibility of DNA to react with MNNG.

The replicating machinery, e.g. DNA polymerase, is not thought to be a likely site of action.

On the other hand, Baker and Tessman (1968) examine the different mutagenic specificities of MNNG for two bacteriophage, one which has single-stranded DNA, one which has double-stranded DNA, and emphasise that dissimilarity of the strands is not responsible for this difference. Rather, it is argued that the molecular environment of the replication point, of which DNA polymerase forms a part, primarily determines the fidelity of replication, according to Watson-Crick principles of base-pairing. The actual function of DNA polymerase in relation to base-pairing remains a matter for speculation, but it is nevertheless significant to note that the results of in vitro experiments of MNNG and mammalian DNA polymerase, in the present study, suggest that substrate binding sites of the enzyme are influenced by reaction with MNNG.

It is considered likely, therefore, that the inhibition by MNNG of $[^3H]$thymidine incorporation into DNA of mammalian cells, is due in part to the loss of DNA polymerase activity. This does not exclude other reactions, involving intracellular transport of precursor, or uncoupling of oxidative phosphorylation, as contributory factors in the inhibition process. The alkyl guanidinium compounds - of which MNNG is an example - are known to be potent inhibitors of oxidative phosphorylation in cells (Hall and Palmer, 1969).

It may be recalled that at low concentrations of MNNG the cells recover (temporarily) the capacity to incorporate precursor into DNA. This may be due to replenishment of inactivated DNA polymerase units by fresh units from precursor "stores" in cells in S phase at the time of treatment, or, to the recovery of inhibited G_1 cells to pass normally into S phase. Although no distinction between them can be made from the present study, each alternative poses the problem of how inhibition is avoided in the presence of MNNG, still at inhibitory concentrations in the medium. For example, at 25 μM(initial) MNNG, recovery is evident at 4 hr. (see Fig. 23), when a concentration of at least 5 μM MNNG remains, as judged from MNNG half-life in growth medium (90 min.). This naturally raises the question of penetrability of MNNG within the cells, and whether the cell wall membrane is altered by exposure to MNNG. Certainly, an alteration of cell membrane permeability, with failure of MNNG to penetrate, may account for the observations of Zampieri, Greenberg and Warren (1968). These authors examined bacteriophage-infected Escherichia coli and found that bacteriophage sensitivity to MNNG was reduced at 5 min., as compared to 0 or 3 min., after adsorption and intracellular growth. The alteration of membrane permeability would be the result of infection of the cell by the bacteriophage.

PART IV

GENERAL SUMMARY

IV. GENERAL SUMMARY

It remains to discuss the results of the present study in the context of the known biological activity of MNNG. The potency of the compound as a cytotoxic agent, over other monofunctional agents, is evident from Table 17. This presumably results from the reactivity of the guanido group - most probably with thiol or amino groups as discussed already (III, D.3.c.iv.) - in addition to the alkylating properties of the methyl group, both derived from MNNG (see Fig. 42). Lawley (1966), in reviewing the alkylation of compounds by carcinogens and other agents, adopts molecular size as the criterion of probability that alkylation of a given molecule takes place within a cell. Thus, DNA is alkylated more extensively than RNA, and RNA more than protein (mole for mole). Although methylation of nucleic acids by MNNG is expected *in vivo*, by analogy with the *in vitro* results (Craddock, 1968; McCalla, 1968a; Lawley, 1968), it is possible that guanidination of groups plays an equal, or greater part in the biological activity of MNNG. From the results of the present study, and the reports of others, the influence of surrounding ionic conditions (pH, thiol, amino), in addition to the locality of MNNG within the cell (structures and/or organelles), would be important in the determination of the groups of the cell which are affected. Thus, the analysis for the significant target molecule for reaction with MNNG assumes a qualitative, as opposed to quantitative, nature.

It is not surprising that for an agent with such reactivity, there are cells at different stages of the life cycle (G_1, S and G_2) which are sensitive to MNNG. The sensitivity of cells in S phase was examined further, although only from the standpoint of the gross inhibition of precursor incorporation into DNA and not from kinetic cell cycle analysis. The metabolic inhibition in cells following treatment with MNNG is similar to that following treatment with other alkylating agents. However, in a more detailed examination of the inhibition of precursor incorporation into DNA, it was demonstrated that MNNG inhibited *in vitro* DNA polymerase activity. Yet, there are several objections to the collation of the *in vitro* results with the *in vivo* observation, some of which have already been mentioned in the discussion of the enzyme inhibition. Other features which require to be explained are the persistence of extractable DNA polymerase activity in cells previously treated with MNNG, and also the requirement for preincubation of extract with MNNG in order to demonstrate, satisfactorily, inhibition of *in vitro* enzyme activity. Since the relationship between the *in vitro* and *in vivo* DNA polymerase activities is not known, these anomalies do not negate the probability that the reaction of *in vitro* inhibition occurs also *in vivo*.

Nevertheless, the general nature of the metabolic inhibition must again be emphasised. In respect of incorporation of precursor into RNA or protein, other enzyme pathways may be shown to be inhibited by MNNG but cannot be commented upon in the absence of experimental results.

The two most significant biological activities of MNNG are as a mutagen and as a carcinogen. Bacterial mutagenesis by MNNG is demonstrated in the experiments of Part II, and it may be surmised that here, also, interference with DNA polymerase activity is of importance. Although it has been reported that DNA polymerase determines the fidelity of replication (see Baker and Tessman, 1968), a more passive rôle for the enzyme is proposed by Freese and Freese (1967). These authors believe that pairing by enzyme recognition of individual nucleic acid bases is unlikely, but rather through acceptance of "fit" according to stereochemical considerations involving the deoxyribose-phosphate moiety. That the conformation of the nucleotide strand (Cerda-Olmedo *et al.*, 1968) is the decisive element in mutagenesis by MNNG is given support by the recent work of Singer, Fraenkel-Conrat, Greenberg and Michelson (1968). These authors find that mutagenicity of MNNG for tobacco mosaic virus, or its RNA, is increased when treatment takes place in dispersing solvents, such as formamide, when neither methylation of guanine nor modification of cytosine occurs to a detectable extent. The nature of the mutagenic event is not known, however.

Recent concern (*Lancet*, 1968) that nitrosamine compounds may be formed by the interaction between nitrites and secondary or tertiary amines has arisen from consideration of the wide use of nitrites (or nitrates) as food preservatives. Such preservatives are used for human and animal foods, and indeed toxic herring meal, incriminated in an outbreak of liver disease in ruminants, was found to contain dimethylnitrosamine (DMN) in the range 30-100 p.p.m. (Sakshaug, Sögnen, Hansen and Koppang, 1965). Liver tumours in rats were induced with 5 p.p.m. DMN in the diet (Terracini, Barnes and Magee, 1967). Such concentrations are on a similar level with the 5 μM MNNG observed to inhibit precursor incorporation into DNA (see Fig. 25). If it is accepted that the inhibition of precursor incorporation into DNA is an expression of interference by MNNG with the function of DNA polymerase, then the results of the present study may be thought to invoke somatic mutation as the event responsible for MNNG carcinogenesis.

However, such an interpretation is a gross oversimplication of events and fails to take into account the observed inhibition of precursor incorporation into RNA or protein (Fig. 40). For instance, MNNG may inhibit mammalian DNA-dependent RNA polymerase (cf. Lingens et al., 1967, see III, D.2.b.iii); guanidination of thiol or amino groups of proteins is also likely to occur within the cell, with possible consequences to functional activity. Furthermore, Barnes (1968) draws attention to the delay, between administration and toxic effect, with a number of poisons, such as the nitrosamines. The target molecule which reacts with the poison, or carcinogen, may have a very slow metabolic turnover and the consequences only become apparent after a number of cell generations. Detection of such a target molecule obviously poses a very great problem.

(1) N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) is a potent cytotoxic agent for mammalian cells.

(a) The multiplication of hamster fibroblast monolayers is arrested by MNNG at an initial concentration of 5 μM, or more, in the medium, although recovery of multiplication takes place at 20 μM or less.

(b) An initial concentration in the medium of 25 μM is sufficient to reduce survival of cells exposed as monolayers to MNNG,

(1) (b) for one hour, to 50% (or less) of controls, as judged by subsequent colony formation.

(2) Cells in G_1, S and G_2 phases of the cell growth cycle are sensitive to exposure to MNNG. The sensitive event(s) in G_1 or G_2 is (are) not determined; events in cells of S phase (DNA synthesis) are described below (5, et seq.).

(3) No permanent alteration of growth characteristics is observed among immediate descendants of cells exposed to MNNG for one hour.

(4) Synthesis of DNA, RNA and protein, as judged by incorporation of radioactive precursor, is inhibited in cells after exposure to MNNG. The extent of inhibition is different for each in the lower range of MNNG concentrations (<50 μM) and in decreasing order of inhibition affects DNA > RNA > protein.

(5) Inhibition by MNNG of incorporation of $[^3H]$ thymidine (a pyrimidine) or $[^3H]$deoxyadenosine (a purine) into DNA of cells occurs to the same extent at equimolar MNNG concentrations (around 60% inhibition with 50 μM (initial) in the medium).

(6) Inhibition by MNNG of incorporation of $[^3H]$ thymidine into DNA of cells is not reversed immediately in washed cells reincubated with "conditioned" medium from untreated controls.

(7) Recovery of incorporation of $[^3H]$ thymidine into DNA of cells takes place within 4 hr. after exposure to MNNG at an initial concentration of 25 μM or less. Studies of MNNG half-life in growth medium give values (around 90 min.) which indicate that incorporation recovers in the presence of inhibitory MNNG concentrations. This finding suggests that MNNG penetration and/or activity within the cell is limited in time.

(8) Inhibition by MNNG of incorporation of $[^3H]$ thymidine into DNA of cells in the first hr. of exposure shows a direct relationship to initial concentration of MNNG.

(9) Penetration of $[^3H]$ thymidine into cells, and its phosphorylation to the triphosphate level by 'kinase' enzymes, is not significantly altered from controls by exposure of cells to MNNG at concentrations which inhibit the incorporation of $[^3H]$ thymidine into DNA of cells.

(10) A dose dependent inhibition of DNA polymerase activity in cytoplasmic extracts from untreated hamster fibroblasts occurs when the extract is preincubated with MNNG before assay of activity in an in vitro system. No significant inhibition is obtained when DNA is pretreated with MNNG before use as primer in the in vitro DNA polymerase reaction.

(11) Exogenous thiol groups or DNA primer protect cytoplasmic extracts from untreated hamster fibroblasts against inhibition by MNNG, when included in the preincubation solution along with MNNG. The precise nature of the groups on the enzyme which react with MNNG is uncertain. On the grounds of the known chemical reactivity of MNNG, a guanidination of enzyme thiol groups is favoured.

(12) Studies of DNA polymerase enzyme kinetics, normal and MNNG-inhibited, with cytoplasmic extracts from untreated hamster fibroblasts suggest that MNNG interfers with substrate (DNA) binding sites on the enzyme. The low degree of purity of the enzyme extract and the deviation of the enzyme reaction from simple Michaelis-Menten kinetics preclude determination of meaningful enzyme inhibition, or other, constants.

(13) The guanido group derived from MNNG is potentially important as a biologically reactive agent in cells exposed to MNNG. Its reactivity must be considered as additional to the ability of MNNG to act as an agent of methylation, as shown by other workers.

(14) No conclusion can be made with regard to the reaction(s) significant to the carcinogenic activity of MNNG.

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